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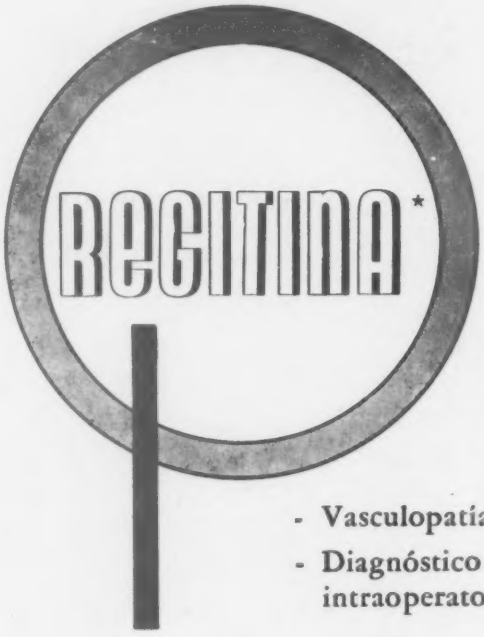

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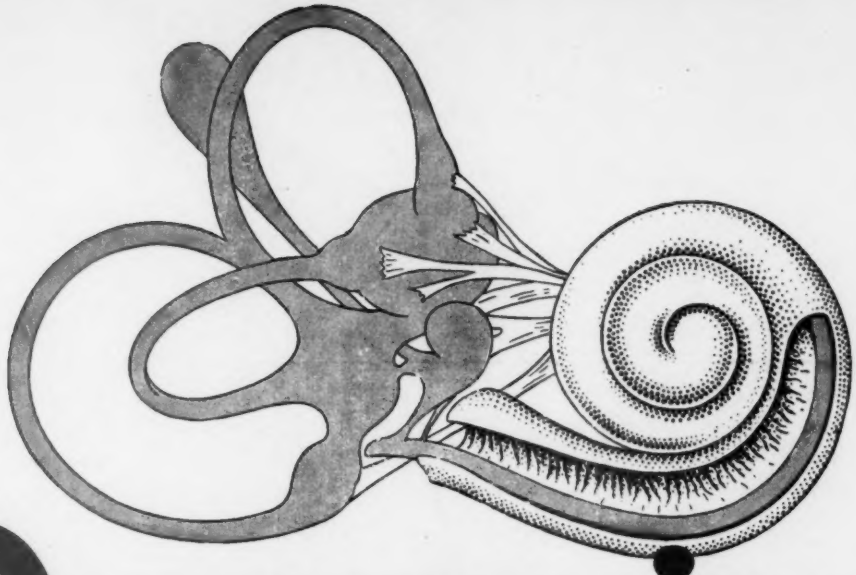

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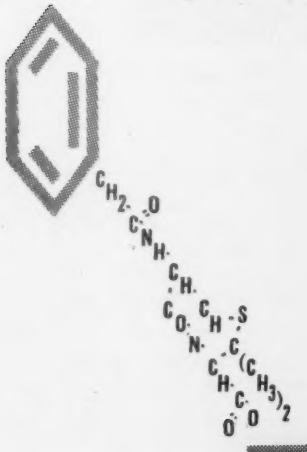

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ASOCIACION CIENCIA E INVESTIGACION
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HORMONAL REGULATION OF THE SEXUAL FUNCTION OF THE MALE TOAD

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SEXUAL FUNCTION OF THE MALE TOAD

Sexual dimorphism. — In the toad *Bufo arenarum* Hensel a clear sexual dimorphism exists (Houssay, Giusti and Lascano González, 1930; Houssay, 1947; Galli Mainini, 1948 d; Burgos, 1950). The male is smaller and generally unicolored, while the female is bicolored (fig. 1). The most typical masculine sexual character are the formations of the corneal layer of the skin: black callosity of the thumb and corneal spicules covering the warts of the dorsum, the skin of the limbs and the glands. Another specific masculine character is that the male croakes when compressed rhythmically behind the axilae. A green band crossing the ventral side from angle to angle of the mandibule indicates by trasparence the pigmented vocal sac in the male. The forearm is thicker and in the sexual epoch the embrace reflex can be elicited in the male by compressing or rubbing the callosity of the thumb or the internal zone of the fore limbe.

Sexual embrace. — At the time of reproduction, which occurs af the beginning of September, and sometimes at the end of August, the male toads, pale and excited, sing constantly. They embrace the female behind the axilae an are carried by them for hours in search of a pond where they remain floating. From time to time the males tighten their embrace and lower their head. During the embrace it is difficult to free them from the female, and they can be pinched and even cut without loosening the tonic sexual embrace.

During the embrace spermatozoa are liberated within the testicles (spermation) and pass into the kidney through the *vasa efferentia testis* (fig. 2). They penetrate in some nephrons and may be seen in the tubuli contorti or around some glomeruli (De Robertis, Burgos and Breyter, 1945). They are then liberated with the urine through the

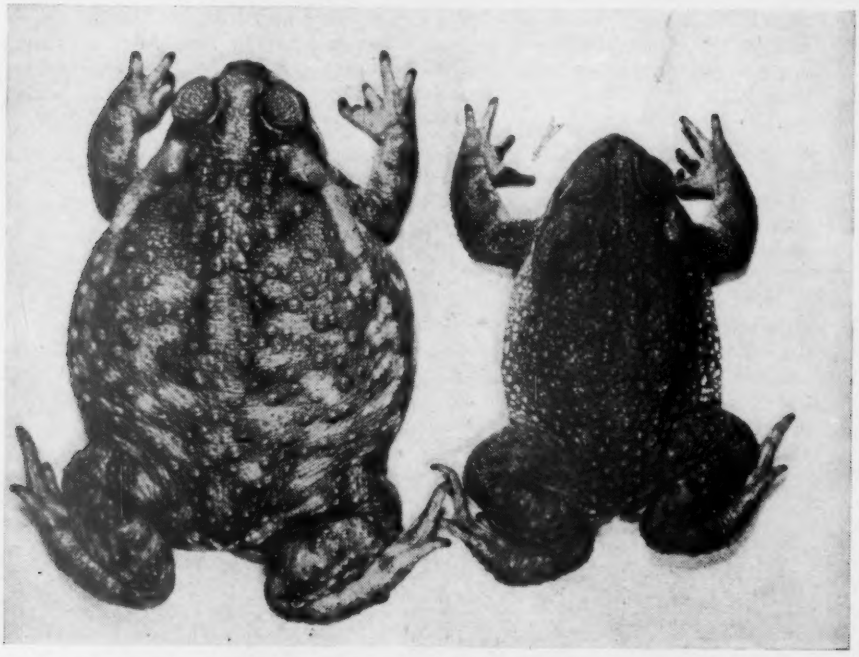

FIG. 1. — *Bufo arenarum* Hensel. *The arrow indicates the callosity of the thumb.*

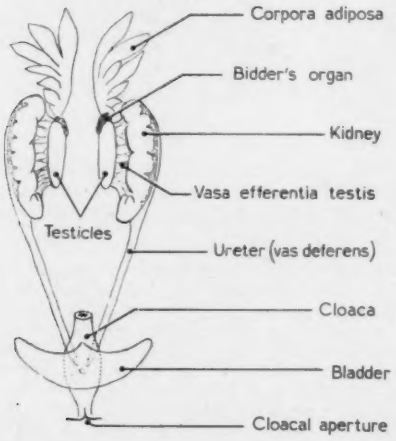

FIG. 2. — *The reproductive organs of the male Bufo arenarum* Hensel.

ureter (ductus deferens) into the cloaca and then to the urinary bladder. From time to time the male expels urine loaded with spermatozoa which fertilize the eggs contained in two long gelatinous tubes which hang from the cloaca when the female ovulates and puts the eggs when being embraced. This phenomenon of external ovulation normally takes place in the water where the female goes before or after the embrace.

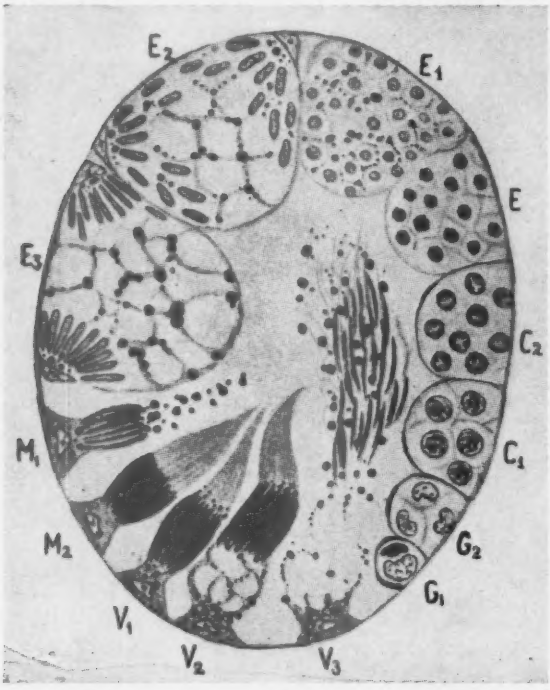

FIG. 3. — *Schematic representation of a testicular ampoule showing the spermatogenetic wave and the relation of spermiation with mucopolysaccharides (in deep black.* G1: *spermatogonia with mucin;* G2: *spermatogonia II;* C1: *spermatocyte I;* C2: *spermatocyte II;* E: *spermatids;* E1: *beginning of vacuolization of the spermatide cyst: drops of mucin and intercellular vacuoles;* E2: *evolution and migration of spermatids; drops of mucin in the center of the vacuolized cyst;* E3: *formation of "bushes" of spermatozoa, central drops and vacuoles; the cyst begins to disintegrate;* M1: *recently formed spermatozoa "bushes"; drops of mucin in the protoplasm of the Sertoli cells and in the lumen of the ampoule due to disintegration of the follicular cyst;* M2: *quiescent Serboli cells with adhered bushes of mature spermatozoa;* V1: *beginning of spermation: vacuolization of the cytoplasm of Sertoli cells; reappearance of mucin drops;* V2: *the vacuoles have grown in the cytoplasm of Sertoli cells;* V3: *outburst of the vacuole, disintegration of the apical portion of the Sertoli cells and expulsion of the spermatozoa surrounded by drops of mucin (R. E. Mancini, M. H. Burgos: Rev. Soc. argent. Biol., 1948, 24, 318).*

Spermatogenesis and Spermiation. — The histological phenomena of spermatogenesis and spermiation have been the object of various studies (Burgos and Mancini, 1947; 1948; Burgos, unpublished). The same may be said of the role of mucopolysacharides in this process (Mancini and Burgos, 1948).

Spermatogenesis takes place within the seminiferous tubes or ampoules in nodular formations called spermatocysts covered by a membrane of flattened follicular cells (fig. 3). Each spermatocyst matures independently and not simultaneously in the same or different seminiferous tube. The mother cell is the primary spermatogonia which lays on the basal membrane; this cell is transformed in secondary spermatogonia and then by division in primary and secondary spermatocytes and there in spermatids. The spermatids nucleus elongates and then by disolution of the intercellular cement are freed in the spermatocyst (fig. 3, E2) and displace themselves in order to adhere to a Sertoli cell (fig. 3, E3).

At that moment the cyst becomes vacuolized. The follicular membrane disintegrates and the cyst vanishes. The heads of the spermatozoids are inserted in the cytoplasm of the distal portion of Sertoli cells near the nucleus and their tail floats in the lumen of the tubules, giving a picture of a brush (De Robertis, Burgos and Breyter, 1945).

A mucopolysacharide can be detected in the cytoplasm of primary spermatogonia which disappears in the secondary spermatogonias. It reappears in drop form between the spermatids (E1 to E3, fig. 3) when an intercellular vacuolization takes place separating the spermatids which disperse (fig. 3, E2) emigrate and insert themselves in the Sertoli cells (fig. 3, E3). When the cyst disintegrates and the young spermatozoa are implanted in the Sertoli cells, these cells show mucin drops in their protoplasm (fig. 3 M1). Later on the mucopolysacharide apparently disappears from the Sertoli cells in which are inserted mature spermatozoa (fig. 3, M2). Under the action of gonadotrophins mucopolysacharide drops reappear in the Sertoli cells (fig. 3, VI); vacuoles are formed which swell and burst leaving the spermatozoa free (V3) while the Sertoli cell remains deflecated and adhered to the basal membrane (Mancini and Burgos, 1948).

The mucopolysacharide exists whenever fluid accumulates, as during intercellular vacuolization within the spermatocyst with liberation of spermatids. It also appears in the Sertoli cell when this cell swells, becomes vacuolized and disintegrates liberating the spermatozoa. This phenomenon initiates spontaneous spermiation (endogenous gonadotrophin) or the spermiation caused by gonadotrophin injection (exogenous) (Mancini and Burgos, 1948). The seminiferous tubes distend themselves by accumulation of fluid in which float the free spermatozoa (Houssay and Lascano González, 1929; Burgos and Mancini, 1947; Mancini and Burgos, 1947).

Seasonal variations. — The secondary sexual characters are present all the year round, but reach their maximum development during spring and their minimum during autumn (Burgos, 1950) (fig. 4). The histo-

logical study of the corneal formations (thumb callosity and epidermal spiculae) has been made during the different seasons of the year (Burgos, 1950) .

The weight of the testis varies slightly with a maximum in August,

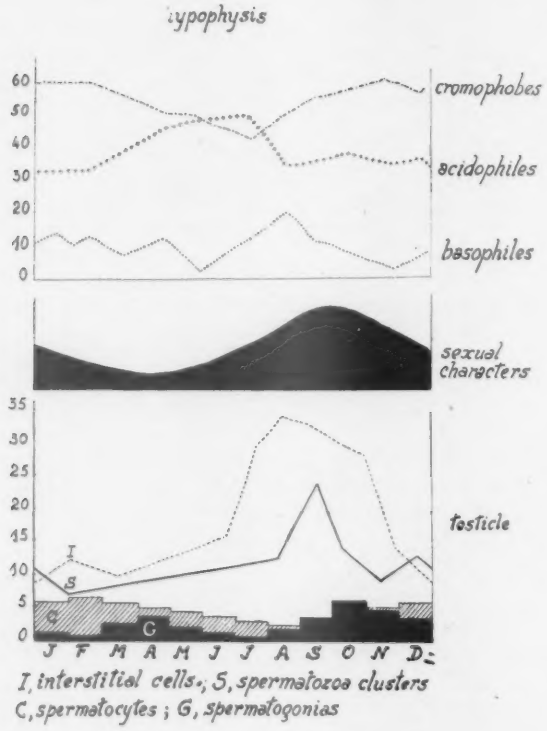

FIG. 4. — *Seasonal variations in the proportion of pituitary* (*J. N. Masselin: Rev. Soc. argent. Biol.*, 1940, *16*, 581) *and testicular cells* (*M. H. Burgos, R. E. Mancini: Rev. Soc. argent. Biol.*, 1948, *24*, 328) *and in the sexual characters* (*M. H. Burgos, R. E. Mancini, Rev. Soc .argent. Biol.*, 1950, *26*, 359).

just before the sexual time, and a minimum in October and November after it is over (Mazzocco, 1940) (fig. 5). The composition of the corpora adiposa has been determined month per month (Mazzocco, 1940) as well as their fatty acid content (Cattáneo, Sutton and Penhos, 1951).

Spermatogenic activity is present all the year round, the spermatogonias predominating in Spring and the spermatocysts and spermatids in Summer and Autumn with a marked increase of the brushes of mature spermatozoa at the end of Winter and beginning of Spring, just before the sexual embrace (Burgos and Mancini, 1948) (fig. 4 and 8).

The interstitial tissue has an atrophic aspect during Summer and is well doveloped in Winter and Spring. The cells have a fibroblastic aspect from January to April and are scarcer. Then their nuclei and the size of the cells increase till June. By amitotic division the number of cells trebbles in July and then increase in size until September-October.

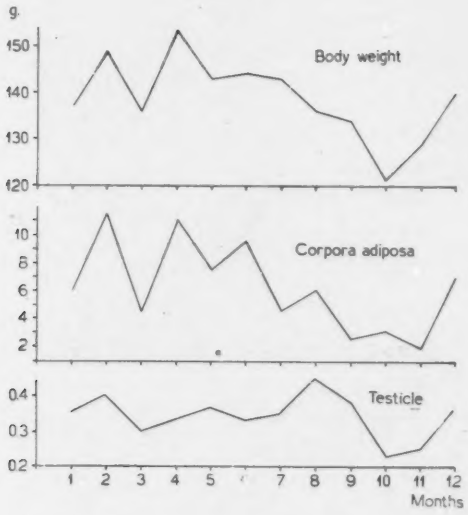

FIG. 5. — *Seasonal variations of body weight and weight of the corpora adiposa and testicle of the toad Bufo arenarum Hensel (P. Mazzocco: Rev. Soc. argent. Biol., 1940, 16, 130).*

The sudanophil and steroid reaction in protoplasm is maximal during the time which corresponds to the sexual embrace. Then the number and size of cells diminishes since November throughout the Summer (Burgos, 1950) (fig. 6).

The greater development of the interstitial tissue parallels the more intense development of the secondary sexual characters (fig. 4). Nevertheless, during Summer the interstitial tissue atrophies while the secondary sexual characters are still well developed. The internal secretion of the interstitial cells is probably sufficient to slow down the diminution of the sexual characters.

The hypophysis also shows seasonal variations. During Autumn and Winter the proportion of granular eosinophil cells increases, with a maximum in July. Then in August immediately before the sexual time the eosinophil cells rapidly diminish and the basophil cells increase in number (Masselin, 1940) (fig. 3). The apparition of carminophil (Masselin, 1940) and azocarminophil (Porto, 1940) cells during the embrace time

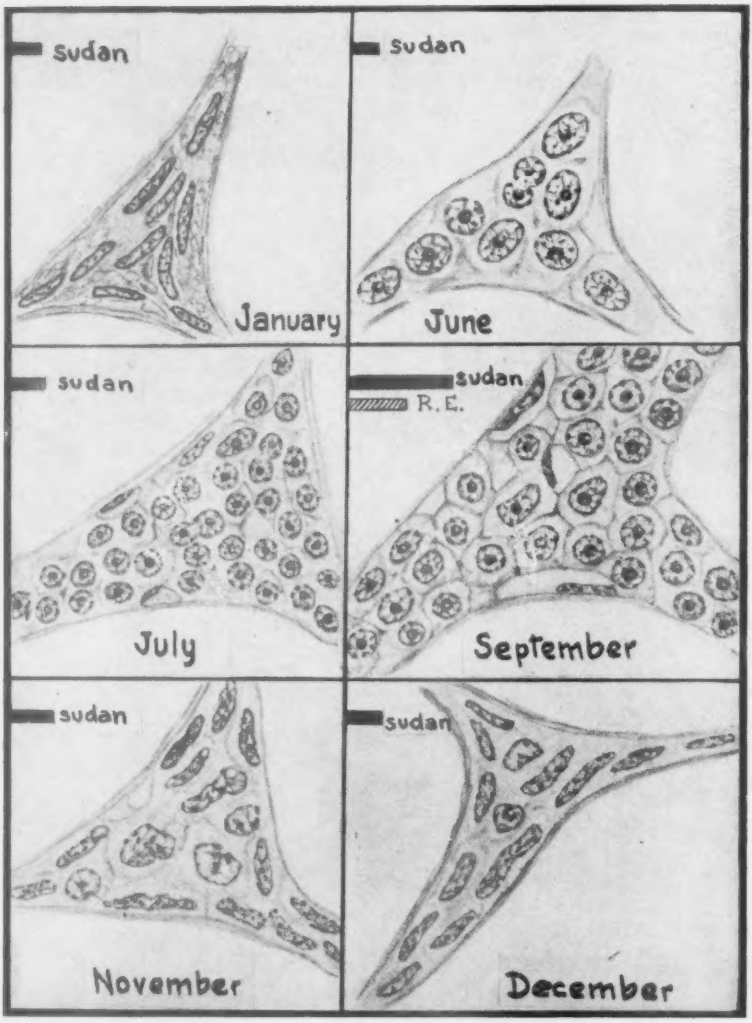

FIG. 6.—*Seasonal changes of the interstitial tissue of the toad's testes. Sudan: sudanophil reaction; R. E.: reaction of the carbonyl groups of lipids (steroids) (M. H. Burgos: Rev. Soc. argent. Biol., 1950, 26, 359).*

has been described, but according to Prieto Diaz and Echave Llanos (1947) the azocarminophil cells are present all the year round.

The gonadotrophic action of the hypophysis has been studied by provoking ovulation or oviposition. It is present during all the year and,

weight for weight, the *pars distalis* of the female is more active than that of the male. There is little variation in potency although two maxima can be detected: one in July and the other in September; and two minima: one in August and another in November-December (Novelli, 1942).

HORMONAL SEXUAL REGULATION

The *pars distalis* of the hypophysis is the primary endocrine motor of the sexual function in both sexes. It secretes gonadotrophin having in the male the following functions:

1) *Trophic action*: a) it contributes to the development and maintenance of the structure and function of the testis. b) the testicular hormones thus secreted contribute to the development and maintenance of the secondary sexual characters: anatomical, functional and of behaviour.

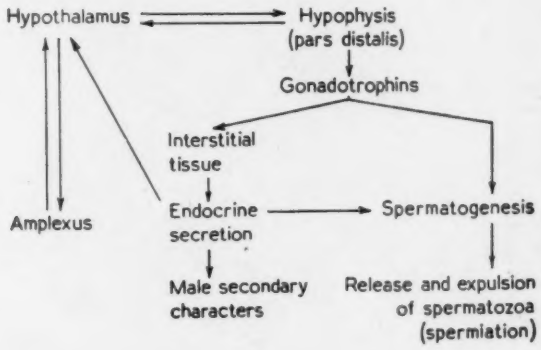

FIG. 7. — *Schematic representation of the sexual function of hypophysis in the male toad.*

2) *Sexual impulse and embrace*: During the sexual period it causes the sexual embrace of the female by the male.

3) *Spermiation*: During the embrace (amplexus) there is a large secretion of gonadotrophin which causes in both sexes the liberation and expulsion of the gametes: spermazoa in the male (spermiation) and ova in the female (ovulation).

In its turn the hipophyseal secretion is influenced by the nervous system through the hypothalamus.

At all times the gonadotrophins secreted by the *pars distalis* maintain spermatogenesis and the endocrine fuction of the testis. This, in its turn, develops and maintains the masculine secondary sexual characters.

During the sexual embrace, probably by way of the hypothalamus, the hypophysis is stimulated and secretes gonadotrophins which cause

the liberation of spermatozoa (spermation) which excreted with the urine fertilize the ova. In the female the sexual embrace causes also the secretion of gonadotrophins, which cause evulation and oviposition, that is expulsion through the cloaca of the ova contained in two long tubes of gelified mucin secreted by the oviduct.

Hypophyso - gonadal interrelation

Hypophysectomy and Testis. — Total hypophysectomy or extirpation of the *pars distalis* (distal lobe) produces a slow and progressive

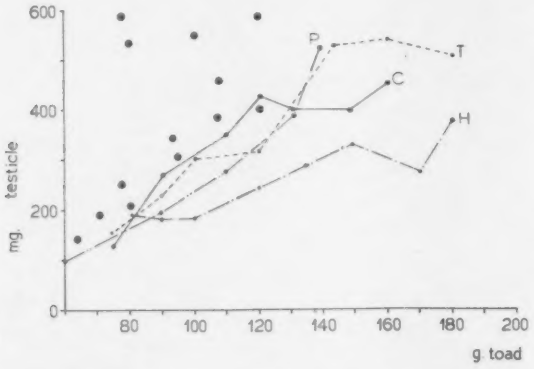

FIG. 8. — *Testicular weight of testicles of toads Bufo arenarum Hensel of different corporal weight. T: controls; H: without pars distalis since 34 to 58 days; C: craneotomized; P: with lesions of the tuber, Black dots; testes of hypophysectomized toads implanted 9 times with pars distalis (1 every 3 days) during 34 days. (B. A. Houssay, J. M. Lascano González: Rev. Soc. argent. Biol., 1929, 5, 77).*

atrophy of the testis and its hypofunction. The testicular weight and volume diminish (Giusti and Houssay, 1923-24; Houssay and Lascano-González, 1929; Houssay, 1949) the atrophy being greater after 2-3 months (Burgos 1949) (fig. 8). This atrophy is due to the lack of pituitary gonadotrophin and not to inanition, which also causes atrophy. Testicular atrophy is found in force fed hypophysectomized toads, in spite of the increase of body weight (Penhos and Cardeza, 1951).

In the absence of *pars distalis* of the hypophysis no compensatory hypertrophy occurs in the small testicular fragment (20mg) left after performing a subtotal castration; while a definite compensatory hypertrophy occurs in the presence of the hypophysis. The testicular fragment grows well in the hypophysectomized toad injected daily during a few days with pars distalis (Houssay and Lascano-González, 1935).

Bidder's organ atrophies in hypophysectomized male toads, castrated or not, while the implantation of *pars distalis* causes its hypertrophy, more marked in the castrated. The implantation of intermediary lobe has no effect. Castration causes hypertrophy of Bidder's organ but this

action does not occur in hypophysectomized toads (Houssay and Lascano-González, 1930, 1931, 1935). Hypertrophy of Bidder's organ and of testicular fragment after castration is less than when only one of these organs is left (Houssay and Lascano-González, 1930).

These experiments show that the hypophysis develops and maintains Bidder's organ and also that its gonadotrophic action is greater after castration. The presence of a testicular fragment or of one Bidder's organ moderates the secretion of gonadotrophins or consumes part of it.

Lesions of the tuber cinereum or craneotomy do not cause testicu-

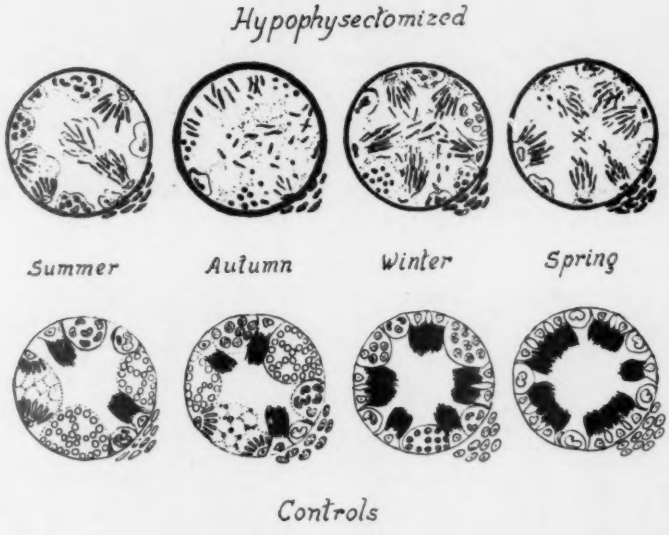

FIG. 9. — *Changes in the testes after hypophysectomy in the toad Bufo arenarum Hensel.* (*M. H. Burgos: Rev. Soc. argent. Biol.*, 1949, *25*, 206).

lar atrophy (Giusti and Houssay, 1924; Houssay and Lascano-González, 1929) (fig. 8).

In the testis of the hypophysectomized toads (or without *pars distalis*) phenomena of progressive atrophy and involution occur very slowly, being evident after 50 to 75 days (Houssay and Lascano-González, 1929; Galli-Mainini and Pinto, 1947; Burgos, 1949) (fig. 8). A marked atrophy of the interstitial tissue occurs, easy to see when it was previously well developed (Winter and Spring) and less apparent when an atrophic aspect was already present (Summer) (fig. 9). Spermatogenesis alters progressively and is at last arrested and picnosis and disintegration occurs. The spermatids are first altered than the spermatocites and later the spermatogonias. The characters of the mucopolysacharide also are altered. The Sertoli cells atrophy and the spermatozoa

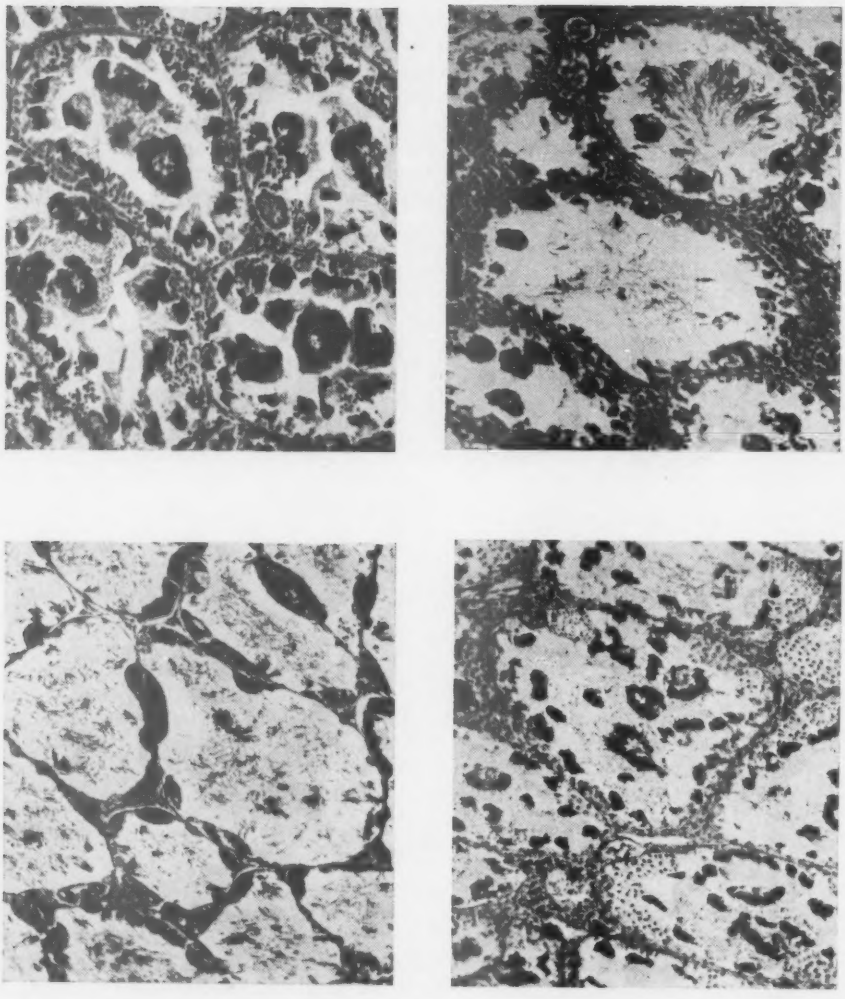

FIG. 10. — *Action of different gonadotrophins on the testis of the toad Bufo arenarum Hensel.* 1) *Normal testis, many "bushes" of spermatozoids, scarce interstitial tissue;* 2) *Pituitary gonadotrophin* (0.2 *mg of dry powder of toad's pars distalis, during* 20 *days*). *Dilated tube with disintegration of the bushes and liberation of spermatozoa. Hyperplasia of the interstitial tissue, open excretory tubes.* 3) *Chronic gonadotrophin from pregnant women's urine* (100 *IU during* 20 *days*): *dilated tube with disintegration of the bushes and liberation of spermatozoa.* 4) *Seric gonadotrophin from pregnant mare's serum* (100 *IU during* 20 *days*). *Hyperplasia of the interstitial tissue.*

brushes separate from them and gradually disintegrate, many free spermatozoa being observed in the seminiferous canals. As a later change the basal membranes increase in thickness (Burgos, 1949).

Action of gonadotrophins. — The gonadotrophins secreted by the hypophysis or the injection or implantation of the *pars distalis* of the

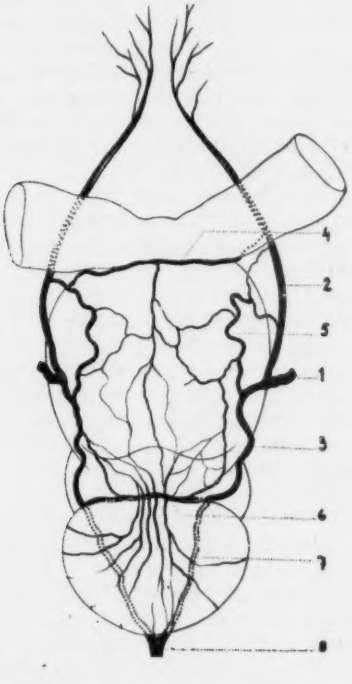

FIG. 11 — *Vascular supply of the hypophysis of the toad Bufo arenarum Hensel (B. A. Houssay, A. Biasotti, R. Sammartino; Rev. Soc. argent. Biol.,* 1935, *11,* 318)

hypophysis have actions which are observed in the following chronological order:

1) *spermiation,* that is, liberation of the spermatozoa within the testis and their subsequent elimination with the urine.

2) *maintenance and stimulation of spermatogenesis.*

3) *maintenance and stimulation of the interstitial tissue* which hypertrophies and increases its secretion.

4) *provocation of sexual embrace,* in the presence of the testis.

5) *development and maintenance of the secondary sexual characters* in the presence of the testis and in its absence.

If injections or implantation of *pars distalis* are repeated daily or every 2 to 4 days, the testicular weight and volume of normal or hypophysectomized toads increase, spermatogenesis is rapidly stimulated and after a few days, the interstitial tissue, the sexual characters and the sexual embrace are also stimulated (Houssay and Lascano-González, 1929) (fig. 10).

Repetition of the injections during 34 days caused great dilatation of the tubes which showed disintegrated brushes and many free spermatozoa floating in their fluid content (Houssay and Lascano-González, 1929) (fig. 11). Great activity of spermatogenesis and increase of cellular elements were also observed (Houssay and Lascano-González, 1929; Houssay, Giusti and Lascano-González, 1929; Burgos, 1951) A very characteristic tissue change was the marked hypertrophy of the interstitial tissue (Houssay, Giusti and Lascano-González, 1929; Burgos, 1951) (fig. 9).

The action of the various gonadotrophins has been compared. The injection of toad hypophysis during several weeks caused marked spermiation, slight stimulation of spermatogenias and a very marked hypertrophy and hyperplasia of the interstitial cells with signs of secretory activity (Burgos, 1951). Chorionic gonadotrophin also caused intense spermiation, the mare's serum gonadotrophin being less active. On the other hand the stimulus of spermatogenesis was greater with seric than with chorionic gonadotrophin. Seric gonadotrophin produced marked proliferation of the interstitial cells but without evident signs of secretory activity; while the administration of chorionic gonadotrophin during an equivalent period of time caused only a slight increase in the number of those cells, which in return showed manifest signs of secretions. The simultaneous administration of chorionic and seric gonadotrophins produced an effect comparable to that obtained by the administration of pituitary gonadotrophin (Burgos and Rufino, 1952) (fig. 9).

Chorionic and seric gonadotrophins injected during prolonged periods of time to hypophysectomized toads had a stimulant action on the three fundamental testicular elements: the germinal cells, the Sertoli cells and the interstitial cells. Hypophysectomy did not alter the spermiation response for many days (Galli Mainini and Pinto, 1947) but diminished it later on (Houssay, Pinto and Burgos, 1953).

Action of sexual hormones on the hypophysis. — No changes in the *pars distalis* were seen 90 days after castration: weight, ocytocic action of the neuro intermediate lobe, ovulatory action of *pars distalis* were the same (Novelli, 1932).

The administration of estrone during only 14 to 20 days did not modify the gonadotrophic (ovulatory) action of the toad's hypophysis (Freire, 1937).

The administration of large doses of estradiol benzoate during long periods of time (58 to 83 days) caused pituitary changes. The basophil cells of the *pars distalis* diminished in number and size, the cytoplasm became degranulated and homogeneous vacuolisation and nuclear irregularities being often observed. The weight of the testicle diminished and all phases of spermatogenesis were altered; the Sertoli cells, reduced in size; disconnected themselves from the brushes and free isolated

spermatozoa were observed in the tubular lumen; picnosis and chromatolysis of the spermatic and the Sertoli cells were observed as well as marked atrophy of the interstitial cells with pigment accumulation and thickening and hyalinization of the tunic of the tubular wall. The Bidder's organ underwent profound involutive alterations (Burgos, 1953). The injection of active doses of *pars distalis* was unable to produce spermiation in some of those toads (Houssay, Penhos and Burgos, 1953).

Hormonal regulation of the secondary sexual characters

Hypophysectomy. — Extirpation of the hypophysis (or of its *pars distalis*) causes, besides testicular atrophy, the regression of the masculine sexual characters: callosity of the thumb, corneal spicules, spontaneous or reflex sexual embrace (Houssay and Giusti, 1930).

Injections of hypophysis. — The daily implantation of *pars distalis* caused after several days the spontaneous or reflex sexual embrace in normal but not in castrated toads (Houssay and Giusti, 1929); the same effect is obtained in hypophysectomized noncastrated animals (Houssay and Giusti, 1930). The *pars distalis* of *Bufo arenarum* produced the embrace in male *Bufo D'Orbigny* (Houssay, Giusti and Lascano-González, 1929) and *Xenopus laevis,* the latter embraced the equally injected ovulating females and fertilized their ova (Shapiro, 1953).

In all these experiments the implantation or injection of *pars distalis* stimulated the testes, their endocrine secretion stimulating the central nervous system and causing the embrace in the male toad (Houssay, Giusti and Lascano-González, 1929).

The injection of *pars distalis* of *Bufo arenarum* caused the apparition of the nuptial apendages (brachial pads) of *Xenopus laevis* (Shapiro, 1943).

Castration and restitution. — Castration caused a gradual and profound regression of the secondary sexual characters and of the sexual embrace, which were apreciable at 20 days, clear cut at 30 and profound at 60 days. The croaking reflex did not disappear (Burgos, 1950).

Testosterone (100 ug) caused after 15-20 days reappearence of the callosity of the thumb and of the corneal spiculae in the castrated toads. Chorionis gonadotrophin and pituitary *pars distalis* were inactive.

In the normal toads only the injection of great amounts of testicular tissue (1 or 2) provoked the sexual embrace (Houssay and Giusti, 1930). In the castrated toad this efect has not been obtained with testosterone or *pars distalis* alone, but a positive effect can be obtained by the simultaneous injection of both substances (Burgos, 1950).

Diencephalo - hypophyseal interaction

The only purely diencephalic phenomenon found in the toad is the sexual embrace, which in many males follows the cauterization of the infundibulotuberal region, from the optic chiasma to the hypophysis by means of a hot needle or the galvanocautery. The embrace is a nervous phenomenon since it is observed in hypophysectomized or castrated animals (Houssay and Giusti, 1930).

Nevertheless the infundibulotuberal lesions are followed by alterations of the hypophysis, because it interrupts the vessels that carry the blood from the tuber to the *pars distalis* (Houssay, Biasotti and Sammartino, 1935) (fig. 11). The circulation stops and an anemic infarct develop in the central and ventral parts of the *pars distalis* (fig. 12). This reaches its maximum in 7 days, lasts some 11 to 17 days and finally the lobe regenerates in 25 to 35 days, the chromophobe cells appearing earlier than the chromophile cells (Houssay and Giusti, 1930; Lascano-González, 1935).

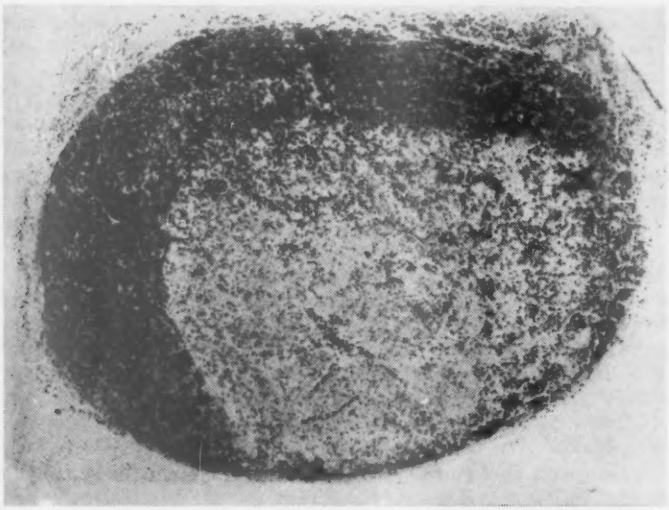

FIG. 12 — *Anemic infarct of the pars distalis of the toad's hypophysis, 7 days after transversal cauterization of the infundibular lobes and tuber cinereum. Central and posterior necrosis (J. M. Lascano González, Rev. Soc. argent. Biol., 1935, 11, 309).*

As a result of the infarct there is an early stage of a few hours of increased reabsorption of glandular products followed by a later stage of slight or marked pituitary hypofunction (Houssay, Biassotti and Sammartino, 1935). The initial increase in reabsorption is characterized by: a) ovulation and ovoposition (Giusti and Houssay, 1922; 1923 and 1924; Houssay, 1923, 1926, 1936, 1947; Houssay and Giusti, 1930; Houssay, Biasotti and Sammartino, 1935); b) spermiation, that is, liberation and expulsion of spermatozoids (Houssay, 1947). These phenomena are not observed after infundibulotuberal lesions in toads in which the *pars distalis* has been removed.

In most toads with infundibulotuberal lesions no testicular atrophy is observed (Giusti and Houssay, 1924; Houssay and Lascano-González, 1929).

The nervous system on its turn influences the hypophysis. The sexual embrace causes the secretion of gonadotrophin by the *pars distalis* of the hypophysis, which provokes sexual erotization and spermiation in the male and ovulation and ovoposition in the female. This ovulation does not occur during sexual embrace in hypophysectomized female toads (Houssay, 1934, 1936). The mechanism by which the nervous system acts upon the *pars distalis* is still unkwown, although partially studied (Houssay and Giusti, 1930).

Spermiation

Definition. — It consists in the liberation of the mature spermatozoa of its insertion in the Sertoli cells; the spermatozoa become free and are found floating in the fluid which fills the semiferous tubes, from where they are then expelled from the testis. In the toad *Bufo arenarum* Hensel this process of liberation and ejection of the spermatozoa is due exclusively to the action of endogenous or exogenous gonadotrophins. The term spermiation, introduced by Van Oordt and Klomp (1946) for this process, is analogous to the term ovulation which designate the discharge of ova by the ovaries. By extension the term spermiant could be applied to those substances which produce spermiation.

The discharged spermatozoa pass through the *vasa efferentia testis* which go from the testis to the kidney, run through some nephrons and come out with the urine by the ureter (*vas deferens*) to the cloaca. Urine accumulates in the bladder and from time to time is expelled with the spermatozoa its contains through the cloacal orifice (fig. 2).

The route followed by the spermatozoa in the spontaneous spermiation during the embrace as described long ago in european frogs by Zwammerdam (1752) and Bidder (1846), etc. according to Gaupp (1904) who gives a synthesis of anatomical and functional date. They were studied by Rugh (1937, 1939, 1941) in the northamerican *Rana pipiens* injected with pituitary.

History. — The liberation of spermatozoa and their individual separation in the fluid of the seminiferous tubes by action of the pituitary gonadotrophin was first described by Houssay and Lascano González (1929) and then widely confirmed (Houssay, Giusti and Lascano González, 1929; Rugh, 1937; Houssay, 1947; Burgos and Mancini, 1947). The mechanism of this process, which is a hydropic vacuolization of the Sertoli cells with disjunction of its apical cytopiasm bringing the liberation of the spermatozoids, was discovered by De Robertis, Burgos and Breyter (1945)) (fig. 3). The role of the hydration of the mucopolysacharides in the vacuolization of the Sertoli cell and the liquefaction of the cement joining the heads of the spermatozoa between themselves and the Sertoli cell was proved by Mancini and Burgos (1948) (fig. 3). The action of gonadotrophins is a direct one, since similar changes occur in the explanted testis *in vitro* as shown by Burgos and Mancini (1947). The passage of the spermatozoa from the testis to the kidney and thence to the urinary tract was studied by De Robertis, Burgos and Breyter (1945).

The early diagnosis of pregnancy by the spermiation of the toad

after injection of pregnant woman's urine is due to Galli-Mainini (1947).

The study of spermiation of the *Bufo arenarum* Hensel has pursued since 1947 by Galli-Mainini, Houssay (B. A.), Burgos, Mancini, Cardeza, Penhos, Pinto, Hartmann, Houssay (A. B.), Valle, etc. in Buenos Aires; Orias, Allende, Astrada, Caligaris and Flores in Cordoba; Hutz in Porto Alegre, without taking into account the numerous studies on the application of Galli-Mainini's test of pregnancy.

Active substances. — The only subtances which produce spermiation in *Bufo arenarum* Hensel are the pituitary and placentary gonadotrophins. In this toad adrenalin and other smypathomimetic amines which produce spermiation in *Leptodactylus ocellatus* and other batrachian (Houssay and Burgos; 1953) are not active nor are some steroids which produce spermiation in *Xenopus* (Houssay, Penhos and Burgos, 1953).

Sodium oxalate produces spermiation in few cases (3/20) in *Bufo arenarum* Hensel (non published experiments of Tabarelli Neto conformed by Penhos).

The active part of the pituitary gland is the *pars distalis*, the neurointermediate lobe being inactive. Spermiation is produced in *Bufo arenarum* Hensel by the pituitary glands of *Bufo arenarum* Hensel of both sexes (Houssay and Lascano González, 1929) those of other batrachians are also active, such as: *Leptodactylus ocellatus* (L.) Gir. (Houssay, 1947; Galli-Mainini, 1948); *Rana pipiens* (Houssay, 1950); *Xenopus laevis, Bufo paracnenmis, Bufo marinus, Bufo d'Orbigny.*

Reciprocally the *pars distalis* of pituitary glands of *Bufo arenarum* Hensel produced spermiation in: *Leptodactylus ocellatus* (Houssay 1947), *Bufo marinus* (Valle, Penhos and Houssay, 1952); *Rana pipiens* (Houssay, 1950), *Xenopus laevis* thus treated embraces the female also injected with *pars distalis* of *Bufo arenarum* and fertilized their eggs which gave rise to tadpoles. (Shapiro, 1943).

Crude extracts of pituitary glands from ox, sheep, swine, dog, guinea pig, cat and fishes did not produce spermiation in *Bufo arenarum* (Houssay, 1948, d). There were some positive results with high doses of pituitary extracts from rat and man (Houssay, 1947) and rabbit (Galli - Mainini, 1948, d). The results may be attributed to the fact that gonadotrophin is not extracted in sufficient concentration from whole hypophysis or is not well absorbed or is inactivated. Actually, when gonadotrophins are extracted from those pituitary glands, activity was demonstrated in the case of sheep (Houssay, 1947, 1948), cattle (Galli-Mainini, 1948, Schweitzer and Bas, 1948, c), horse (Galli-Mainini, 1948; Schweitzer and Bas, 1948, c) and pregnant mare (Schweitzer and Bas, 1948, c).

Spermiation has been obtained with pure follicle-stimulating hormone and with luteinizing hormone prepared by Li and McShan from sheep's hypophysis; the association of both hormones potentiates their action (Houssay, 1947, 1948, 1949, 1951).

Urine from women castrated or in menopause have yielded negative results (Houssay, 1947; Galli Mainini, 1948).

The following placental gonadotrophins produced spermiation: a) pregnant women's urine (Galli Mainini, 1947; Houssay, 1947; Schweitzer

and Bas, 1948 a, b); b) human placenta (Galli-Mainini, 1948 c); c) pregnant mare's urine (Galli Mainini, 1947; Houssay, 1947; Schweitzer and Bas, 1948 a, b) mare's placenta (Schweitzer and Bas, 1948 c).

Mechanism. — The histological mechanism has been described under "Spermatogenesis and spermiation" and is represented in fig. 3. In the apical portion of the Sertoli cells drops of mucopolysacharides appear, then vacuoles develop, there is swelling of the cells; their apical portion disintegrates and the spermatozoa free themselves when the cement which joins their heads disintegrates. The free spermatozoa fall in the lumen of the seminiferous tube which dilates markedly due to the accumulation of fluid. The testis, increased in size, is more translucid and becomes congested. The *vasa efferentia testis* are also dilated by the spermatozoa containing fluid. The spermatozoa pass into some nephrons and thence with the urine into the cloaca and are deposited in the bladder. This histological mechanism was described by De Robertis, Burgos and Breyter (1945) and the role of the mucopolysacharides by Mancini and Burgos (1948).

Gonadotrophins exert their action directly in the testis. Spermiation is obtained in toads deprived of some of the following organs: brain, lung, digestive tract, liver, pancreas, thyroid, spleen (Houssay, 1947); it can be obtained with subnormal amounts of chorionic gonadotrophin in hepatectomized toads (Galli Mainini, 1949). In toads without hypophysis spermiation can also be elicited, but after 60 to 78 days greater doses of gonadotrophins are needed (Galli Mainini and Pinto, 1947; Houssay, 1947; Flores, 1948); in hypophysectomized toads estimulation of spermatogenesis and development of interstitial tissue are also obtained (Houssay and Lascano González, 1929; Galli Mainini and Pinto, 1947).

Spermiation is also elicited by injection of gonadotrophin into the testes *in situ* or applying it in explanted testicular slices maintained in Holtfreter fluid. The same histological changes in the Sertoli cells occur as in the intact animal. Pituitary and chorionic gonadotrophins have a more rapid action than seric gonadotrophin. (Burgos and Mancini, 1947 a, b; Mancini and Burgos, 1947 a, b). To produce spermiation 0.1 to 0.3 I U of chorionic gonadotrophin in 0.3 ml are enough.

Transport of spermatozoa seem to be effected, under the action of gonadotrophins, by the fluid which dilates the seminiferous tubes. No evidence has been found of muscular relaxation in the *vasa efferentia testis*, the dilatation of which is visible when the spermatozoa containing fluid circulates through them.

Celerity and duration of the effect. — In toads with good diuresis spermatozoids have been found in the ureter some times 10 minutes, and in a third of cases 15 minutes, after the intravenous injection of high doses of pituitary gonadotrophins (Houssay, 1947). In the cloaca they are found after 30-60 minutes in increasing amounts. These results were obtained at 23° C but at lower temperatures intervals are prolonged. (See "Temperature").

Spermatozoids may be found in the urine during variable periods.

At 23° C the maximal reaction occurs at about 2 to 5 hours. With small doses the spermatozoa persist a few hours, with greater doses they may persist even 50 hours (Galli-Mainini, 1948), 2 days and in one case 3 days (Sammartino and Arrighi, 1948). It is commendable not to use again a toad until after one week following a positive reaction.

Specificity. — Spermiation in the the toad has not been obtained with any other chemical substance or hormone apart from the placentary and pituitary gonadotrophins. No action was obtained with: a) *tissues of toad or cattle*: liver, kidney, adrenals, spleen, heart, muscle, thyroid, testis, ovary (Houssay, 1947); b) *urine of women castrated or in menopause* (Galli Mainini 1947; Houssay, 1947; c) *urine of other*

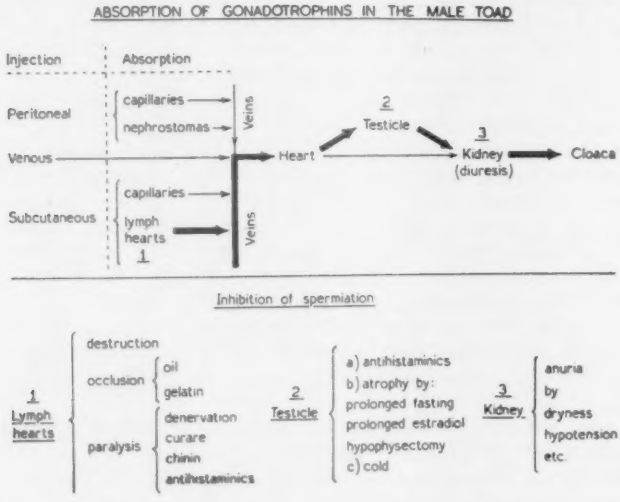

FIG. 13 — *Routes of absorption of gonadotrophins in the male toad.*

pregnant females: rat, cow (Houssay, 1947); d) *serum of other pregnant females*: cow (Galli-Mainini, 1948), cow, sheep (Mayer and *et al*, 1947); e) *sex hormones, natural or synthetic*: estradiol, estrone, stilbestrol, ethynil testosterone, progesterone, testosterone (Galli Mainini, 1947; Houssay, 1947; Hutz, 1947); f) *corticoadrenal hormones*: desoxycorticosterone acetate (Galli Mainini, 1947; Houssay, 1947), cortisone, corticosterone; g) *thyroid hormone*: thyroxine (Galli Mainini, 1947); h) *pancreatic hormone*: insulin; i) *pituitary hormones*: adrenocortico trophin, somatotrophin, thyrotrophin; j) *various chemical substances*: (see fig. 16); k) *enzymes;* hyaluronidase, papain, trypsin, lisosyme.

Urine of castrated or menopausic women did not produce spermiation even after concentration of gonadotrophins by various methods: alcohol, or acetone or tannate precipitation, caolin adsorption, etc., etc.

Some sympathicomimetic substances produce spermiation in *Rana pipiens, Rana esculenta, Xenopus laevis*, etc., but not in *Bufo arenarum*

Hensel even in subcutaneous doses of 10 mg. Among the substances which produced spermiation in *Leptodactylus ocellatus* (L) Gir. are: adrenalin, noradrenalin, isuprel, levo-cobefrin, m-oxi-norephedrine, etyl-N - noradrenaline, epinine, arterenone (Houssay and Burgos, 1953).

Routes of administration. — Gonadotrophins are not absorbed through the skin nor by the digestive tract (Galli Mainini, 1948; Valle, Penhos and Houssay, 1952). By intracardiac or intravenous injection they produce positive results somewhat more rapidly (Galli Mainini, 1948; Mancini and Burgos, 1947). Subcutaneous administration by dorsal route produces identical number of spermiations as the intravenous route, while the intraperitoneal route is less efficient and the intramuscular (thigh) and ventral subcutaneous give slower results, and in small or medium doses give a slightly lesser proportion of positive results (Valle, Penhos and Houssay, 1952).

Absorption of dorsal subcutaneous injections is effected principally through the lymph hearts and to a much lesser proportion through the blood capillaries. If the 4 lymph hearts are destroyed a dose previously active is ineffective; but if 3, 2 or even one lymph heart is kept intact absorption takes place. Destruction of the 4 lymph hearts prevents the absorption of gonadotrophins injected into the thigh, but not that of those injected into the peritoneal cavity. This is due to the fact that intraperitoneal absorption (see fig. 13) takes place through ciliated funnels which open in the kidney surface (nephrostomes) and carry the peritoneal fluid to the veins; a small proportion of peritoneal absorption is effected through the blood capillaries.

Nevertheless, if in toads with the 4 lymph hearts destroyed, pituitary or chorionic gonadotrophins in doses 25 times greater than those normally are active subcutaneously, absorption of gonadotrophins in amounts sufficient to produce spermiation occurs. In this case the absorption takes place through the blood capillaries (see fig. 13) (Valle, Penhos and Houssay, 1952).

The action of the lymph hearts may be abolished by various means: a) *destruction;* b) *occlusion* of their entrance orifices by oil (Valle and Paraventi, 1950; Valle, Penhos and Houssay, 1952) or gelatin (Valle, Penhos and Houssay, 1952); c) *paralysis*: by denervation or destruction of the spinal cord (Valle, Penhos and Houssay, 1952), by curare (Valle and Paraventi, 1952; Hartmann,1953), chinine (Allende and Caligaris, 1949; Houssay, Penhos and Burgos, 1953), neoantergan and acids (Hartmann, unpublished).

Doses. — With the same dry powder of pituitary *pars distalis,* intravenously injected, spermiation and appearance of spermatozoa in urine occured in variable proportion according to the seasonal sensitivity (see table).

The *pars distalis* of *Leptodactylus ocellatus* and *Xenopus laevis* produced spermiation in *Bufo arenarum* in doses of 0.05 mg or more of dry powder (Houssay, 1951).

With commercial preparations of chorionic gonadotrophins spermiation was obtained in 2/3 of cases with 38 I U (Schweitzer and Bas, 1948 a) or 40 I U (Galli Mainini, 1948, 1949). Using international standard chorionic gonadotrophin 50 % of positive results were obtained with 22,5

TABLE I

Dry powder of "pars distalis" of "Bufo arenarum", Hensel (mg)

	Lots of 12 toads					
Mg, dry *pars distalis*...	0.05	0.04	0.03	0.025	0.02	0.01
Exp. 1						
Positive per cent	100	100	—	—	60	50
Exp. 2.						
Positive per cent	100	100	63	55	25	0

I U, 66% with 27 1 U and 100% with 35 I U. (Houssay, 1949). In the numerous experiments of fig. 14 the dose giving 66 % of positive results varied according to the season between 20 and 32 I U (average 24.5 I U) and the dose giving 50 % between 22,5 and 36 I U (average 29).

Males are more sensitive than females. With *pars distalis* spermiation is produced following doses 4 or 5 times smaller than those necessary for producing ovulation. With chorionic gonadotrophin the difference is even greater: spermiation follows doses 35 to 100 times smaller than those necessary for producing ovulation. (Houssay, 1947, 1949, 1951; Galli Mainini, 1948).

Schweitzer and Bas (1948, a) called *Bufo unit* (B U) they meant of course *Bufo arenarum* unit the amount of gonadotrophin, of whatever origin, which produces spermiation in 2/3 of the injected toads and established the equivalence of this unit with the international unit (Table II) as determined in rats (1948, b; 1951).

TABLE II

Equivalence of Bufo arenarum unit and international units of gonadotrophins

	Bufo unit		I U
Chorionic gonadotrophin	1	equals	38
Pregnant mare's serum	1	„	150
Pregnant women's serum	1	„	50
Pituitary gland of pregnant mare	1	„	28
Pituitary gland of horse	1	„	17
Mare's placenta	1	„	25
Pregnant women's serum (not extracted)	1	„	13
Pituitary gland of bovine	1	„	0.6

The toad is 4 times more sensitive to chorionic (pregnant women's urine) than to seric (pregnant mare's serum) gonadotrophins and 63 times more sensitive to pituitary gonadotrophin of bovine origin than to chorionic gonadotrophin (pregnant women's serum, Table II).

Comparative sensitivity of different animals to chorionic gonadotrophin has been established by Cheymol, Henry and Thevenet (1952). The *mouse unit* is the dose which produces a 100% increase in weight of seminal vesicles (2 I U) or estrus (2.51 I U). The *rat unit*, that which produces 100% increase in weight of seminal vesicles (2.5 I U) opening of the vagina (1 to 2 I U), ovarian hyperemia (0.75 I U) increase in weight of ovaries (1 I U). The *rabbit unit* equals 6 to 8 I U. The *Bufo bufo* unit equals 30 to 40 I U. The *Rana esculenta* unit equals 4 to 10 I U and the *Xenopus laevis* unit equals 40 to 70 I U.

According to Hartmann Perdomo and Chapman (1949), *Rana pipiens* give 50% of positive reaction with 5.3 I U of chorionic gonadotrophins. According to Thorborg and Hansen (1951), *Bufo bufo* give 50% of positive reaction with 4.5-5.9 I U in March (beginning of spring) and with 11.5012.5 I U in August (Summer). The *Bufo d'Orbigny,* smaller than *Bufo arenarum* gives positive reaction with smaller doses (Galli Mainini, 1948).

Bufo arenarum of 80 - 120 g of body weight are more sensitive than those of 125 - 175 g and much more that those of 180 to 260 g (A. Houssay, unpublished data).

The rabbits are more sensitive than toads since a positive Friedman test is obtained with 4 I U (Thorborg and Hansen, 1951) or 6-8 I U Cheymol ad coll., 1952). Houssay (1949) obtained 50% of positive results with 15 I U, 66% with 17 I U and 100% with 25 I U. These high values are due probably to the fact that young female rabbits, (1.4 to 1.7 kg) which are less sensitive, were used.

In testes *in vitro* a positive action was obtained with 0.01 to 0.02 I U of chorionic gonadotrophin. Slices of testicular tissue were maintained for 4 hours in 0.1 ml of toad's serum and 0.2 ml of Holfreter fluid at room temperature. They were then fixed, included, cut and colored (Burgos, unpublished).

It is better to administer the dose in a simple injection than to give it in various injections (Houssay, 1947; Galli Mainini, 1947, 1948, Hutz, 1947).

Quantitative determination of gonadotrophins. — Gonadotrophins can be quantitatively determined by the spermiation test in the male toad (Galli Mainini, 1947, 1948, 1949) (Houssay, 1947, 1949, b; Schweitzer and Bas, 1948). The methods of estimation have been specially studied by A. B. Houssay (unpublished). Hartmann-Perdomo and Chapman (1949) have also used *Rana pipiens* and Thorborg and Hansen (1951) *Bufo bufo* and *Rana esculenta* for quantitative estimations. For reliable quantitative estimations 6 toads should be employed according to Schweitzer (1948, 1951) or more (A. B. Houssay).

Conditions depending on the animal

Seasonal variation. — The existence of seasonal variations of the sensitivity of batrachian testes to gonadotrophins has been verified in different species. In some batrachians spermatogenesis stops during some months, while in others only its intensity varies. In *Bufo arenarum* Hensel it occurs all the year round in spite of seasonal variations in sensitivity studied by Burgos and Mancini (1948).

The toads testis are sensitive to gonadotrophins all the year round. Differences in sensitivity have been observed in some seasons (Houssay, 1947, 1951) which according to Galli Mainini (1948, 1949) are only slight. A methodical quantitative study has been made by Dr. J. C. Penhos injecting every month, during 2 years, lots of 10 toads each with one of the following doses: 15, 22.5, 20 and 40 I U of the international standard preparation of chorionic gonadotrophin (fig. 14). The minimal

sensitivity observed in May (middle of Autumn). The reactivity increases rapidly in June an then more slowy and gradually (in Winter) until September, the mating epoch. It remains high in Spring and the maximal sensitivity is observed in November. It begins to diminish in December, continuing in Summer and Autumn until the minimum is reached in May. There is therefore a minimal sensitivity in the middle of Autumn, a maximal sensitivity in the middle of Spring, an increasing sensitivity in Winter and a decreasing one in Summer and Autumn (figure 14).

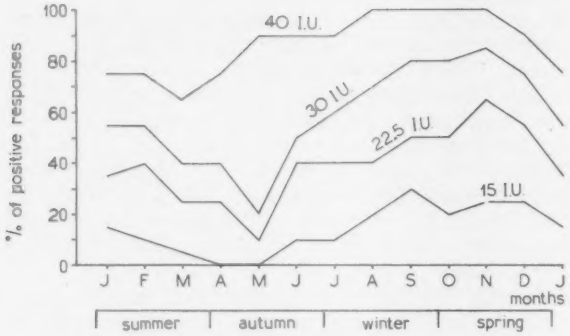

FIG. 14. — *Seasonal variation of the testicular response of Bufo arenarum Hensel to chorionic gonadotrophin (international standard preparation). Ordinates express percentage of positive spermiation in each of the 4 groups of 10-20 toads each month. Doses per toad for each of the 4 groups were: 40, 30, 22.5 and 15 international units. Temperature 23° C* (Penhos, J. C.; *Rev. Soc. argent. Biol.*, 1954).

Size. — Toads sexually inmature do not react to gonadotrophins. Galli Mainini (1948) obtained only 1/3 of positive reactions with toads weighing 70 to 80 g and 69 % with toads of 80 to 99 g. Animals weighing 100 g (80 to 120 g) and with well developed male sexual characters are generally used. Very large toads need larger doses of gonadotrophin (A. B. Houssay, unpublished).

Time of captivity and fasting. — Positive reactions are obtained in toads after 2 to 5 and even 10 months of fast (Galli Mainini, 1948). When inanition is prolonged the testes atrophy, a weight loss of 93 % occuring in 40 weeks. Microscopical observation shows marked atrophy of spermatogenetic and interstitial elements, free spermatozoa in the tubules, scarce brushes, atrophic Sertoli cells, basal membrane thicknened and flexuous (Penhos and Cardeza, 1950, 1951). These animals may become insensitive. Testicular atrophy can be prevented by feeding the toads once or twice a week with calf's liver (Penhos and Cardeza, 1950, 1951). This feeding improves also the atrofied testis.

Hypophysectomy. — Spermiation is obtained in toads even 78 days after hypophysectomy (Houssay, 1947; Galli Mainini and Pinto, 1947;

TABLE III

Influence of temperature and illumination on spermiation in Bufo arenarum Hensel caused by chorionic gonadotrophin. Percentage of positive reaction in each lot (Galli Mainini, 1948-1949).

Dose I. U.	Temperature			Light	Darkness
	30°-32°C (*)	15°-17°C	3°-5°C		
20	22	4	0	0	28
30	20	10	0	7	38
40	92	90	10	64	92
50	90	90	22	77	98

(*) The toad's temperature was 24°C.

Flores, 1948). On occasions, after this time interval the active threshold doses are somewhat higher than in the controls.

Hepatectomy. — In hepatectomized toads spermiation is obtained with smaller doses (Galli Mainini, 1947, 1948).

Adrenalectomy. — Adrenalectomy does not prevent spermiation unless the kidney has been seriously injured or anuria occurs.

Ambiental conditions

Temperature. — Positive reactions may be obtained even at 3° - 4° C, but spermiation is retarded in some cases up to 18 (Hutz, 1947) and 24 hours (Houssay, 1947). It has been obtained in 18 hours at 3° C, in 2hs. af 10°; in 50 min. at 20°; 40 min. at 35°; 30 min. at 50° (Hutz, 1947). The percentage of positive reactions are higher at 30-32°; still high at 15-17° and diminish greatly at 3-5° C. (Table III). Our experiments are usually made at 23° C.

Light. — In the majority of cases positive reactions are equally obtained with the toads exposed to light or in darkness. But with smaller doses of chorionic gonadotrophin a greater proportion of positive results were obtained in toads kept in darkness (Galli Mainini, 1948, 1949) (Table III).

Dessication. — In dehydrated animals urine formation may be arrested, in which case spermatozoa cannot reach the cloaca even when histological spermiation has taken place in the testis. Submersion in water or injection of fluid suffices to induce urine formation and arrival of spermatozoa to the cloaca (Valle, Penhos and Houssay, 1952). The injection of 3 cc of saline, intraperitoneal, is a good practice in all tests.

Substances which modify spermiation

The substances mentioned in Table IV did not produce spermiation

TABLE IV

Substances assayed (dorsal subcutaneous injection for their effect on spermiation or on modifying the action of gonadotrophin (0.05 mg of dry toad's pars distalis by subcutaneous or intravenous route).

Substance	Dose mg	Spermiation	Action on spermiation due to gonadotrophin	Author
Acetylcholine Cl.	10-200	no	—	4,6
Ascorbic acid	500	no	—	4,6
Adrenal extract	3 ml	no	—	6
Adrenalin Cl.	0.1-10	no	no	4,6,8,9
Adrenocorticotrophin	100-250	no	no	6
Aminopterin	5	no	no	6
Antistine	20	no	inhibits	7
Atropine sulf.	1-5	no	no	6
Benadryl	20	no		7
Benzedrine sulf.	1-10	no	inhibits	9,5
Chinine Cl.	10-30	no	no	2,6
Cobefrin	10	no	inhibits	5
Copper sulf. acet.	5-50	no	no	4,6
Cocaine Cl.	1-5	no	—	6
Cholesterol	10	no	—	4,6
Cortisone	10	no	—	6
Curare	—	no	—	11,3 bis
Desoxycorticosterone acetate	5-25	no	inhibits	3,4,8
Dibenamine	30-50	no	no	6
Dihydroxyephedrine	10	no	no	5
Dihydroxyphenylalanine	10	no	—	5
Ephedrine sulf.	5-10	no	—	5,9
Estradiol benz.	see text	no	no	3,4,6
Ethyl-N-noradrenal.	10	no	—	5
Ethinyl-testosterone	10	no	—	4
Fenergan	20	no	inhibits	7
Heparin	5-15	no	no	6
Hialuronidase	3000-5000 U. tr.	no	no	6
Histamine	5-50	no	no	6
Isuprel	10	no	—	5
Luminal	30	no	—	6
Methylene blue	—	no	—	3
m-oxy-norephedrine	10	no	—	5
Neoantergan	20	no	inhibits	7
Neosinephrine	10	no	—	5
Noradrenalin	1-10	no	—	5
Novocaine	5-40	no	no	2 bis
Oxytyramine	10	no	—	5
Papaine	10	no	no	6
Piribenzamine	20	no	inhibits	7
Pitressine	10 U	no	no	3,4,8
Pregnant serum (rat, cow, sheep, pig)	—	no	—	3,4,6
Procaine	3-50	no	no	6
Progesterone	1-10	no	no	3,4,8
Prolactine	20	no	no	3,4 bis
Prostigmine	1-2	no	no	6
Saliva	5 ml	no	no	6
Snakes (venom)	—	no	—	3,6
Somatotrophine	50	no	—	6
Stilbestrol diprop.	see text	no	—	3,4,6
Sulfathiazole	100	no	—	3,4,6
Sympathol	10	no	—	1

Substance	Dose mg	Spermiation	Action on spermiation due to gonadotrophin	Author
Testosterone prop.	5-30		no	5
Tyramine	10	no	—	3,4,8
Tyrotrophin	100 U	no	—	5
Tissues (cow, sheep)	10	no	—	6
Trypsine	—	no	—	6
Ceritol	10	no	—	5
Yohimbine Cl.	1-10	no	—	4
Zinc sulf.	—	no	—	3

The authors mentioned are: 1, Allende, Astrada and Orías, 1948; 2, Allende and Caligaris, 1949; 2 bis, Allende and Orías, 1950; 3, Galli-Mainini, 1947 c, 1948 d; 3 bis, Hartmann, 1953; 4, Houssay, 1947 a, 1948 b, 1949 a; 5, Houssay and Burgos, 1953; 6, Houssay, Penhos and Burgos, 1953; 7, Houssay, Penhos, Burgos and Hartmann, 1953; 8, Hutz, 1947; 9, Moussatché, 1950; 10, Valle and Paraventi, 1952; 11, Valle and Paraventi, 1953; for complete reference see the bibliography at the end of the paper.

in the toad. They were also assayed injecting them subcutaneously together with 0.05 mg of dry *pars distalis* of toad's pituitary gland in 1-2 ml of isotonic saline solution to see whether they prevented or diminished the action of gonadotrophin. When inhibition occured the experiment was repeated injecting the gonadotrophin intravenously; if inhibition persisted it was attributed to some action on the testis itself or to inactivation of gonadotrophins in blood; if inhibition disappeared the original result was attributed to a defect in the subcutaneous absorption of gonadotrophin. In cases where inhibition persisted the direct action of the substance was assayed adding it to testis slices explanted in toad's serum and Holtfreter fluid to which gonadotrophins were added, to see whether the action of the latter was also inhibited *in vitro* (Penhos, Burgos and Hartmann, 1953).

A few substances were injected intravenously such as: atropine, histamine, adrenaline, hyaluronidase, papain, adrenocorticotrophin. Gonadotrophin was also injected intravenously in experiments with adrenalin, atropine, antihistaminics, dibenamine and heparin.

Fig. 13 shows the principal mechanisms which may be responsible for spermiation inhibition.

The lymph hearts absorb the fluid injected in the dorsal subcutaneous space and send it to the veins. Absorption by this route is stopped if the action of the lymph hearts is impeded by destruction (cauterization), occlusion (oil or gelatin) (Valle, Penhos and Houssay, 1952), or paralysis due to: a) *denervation* (Valle, Penhos and Houssay, 1952); b) *curare* (Valle and Paraventi 1952; Hartmann, 1953); c) *quinine* (Allende and Caligaris, 1949; Houssay, Penhos and Burgos 1953); antihistaminics (Houssay, Penhos, Burgos and Hartmann, 1953). Intravenous or intraperitoneal injection of gonadotrophin still produces spermiation in toads with lymph hearts paralyzed by curare or quinine.

The antihistaminics can inhibit spermiation induced by gonadotro-

phins by two mechanisms: a) paralysis of the lymph hearts thus impeding the absorption of gonadotrophin injected into the dorsal sac; b) by inhibiting the action of gonadotrophins on the testis itself. The latter mechanism can be proved *in vivo* by injecting gonadotrophin intravenously in toads which have received a subcutaneous injection of antihistaminics or *in vitro* by adding gonadotrophin to explanted testis slices treated with antihistaminics. The direct inhibition is not very marked, but it is clear cut (Houssay, Penhos, Burgos, Hartmann, 1953).

Reaction of the testis to gonadotrophin may be subnormal if the animals have a lower sensitivity due to prolonged inanition, long term hypophysectomy, cold, ect. A prolonged treatment with estrogens may also produce involutive alterations in the testis (Burgos, 1953) and diminish its sensitivity to gonadotrophins (Houssay, Penhos and Burgos, 1952). Cold may retard the action of gonadotrophin and may inhibit the response to low doses.

Anuria due to dissecation (Valle, Penhos and Houssay, 1953), hypotension etc. may be the cause of false negative reactions.

Diagnosis of early pregnancy

History. — The test for early diagnosis of pregnancy based upon the spermiation produced in batrachians by the subcutaneous injection of pregnant women's urine was first found by Galli Mainini in 1947 in *Bufo arenarum* Hensel and should therefore be called *Galli Mainini test.* He also found that other batrachians could be used for the test and very rapidly other authors verified the same in other species common in different countries.

Technique. — After verifying that the urine of the toad to be used in the test does not contain spermatozoa 10 ml of the first urine voided in the morning are injected subcutaneously. In general, when using other species, an amount equivalent to 10% of the weight of the animal should be injected. Not less than 10 ml should be injected in the *Bufo arenarum* except when quantitative determinations are desired. The injection should be done in the dorsal lymph sac introducing the needle in the anterior part and directing it towards the posterior part of the animal or else from one side to another to avoid loss of urine when the toad adopts its normal position.

The ventral subcutaneous route has been used (Pou de Santiago, 1947); González Torres, 1949), but the results thus obtained are somewhat worse than those using the dorsal subcutaneous route (Valle, Penhos and Houssay, 1952).

One toad was employed first for the test; then two and now even three are recomended. For exact quantitative determinations 5 to 10 toads should be used for each dose.

Three hours after the injection of urine the toad is held with its dorsum looking down and a pipette is introduced, 0.5 to 1 cm, into the cloaca, moving its tip to and fro until a small amount of urine is collected. A new sample is obtained after 24 hs. If the reaction is negative nonabsorption of gonadotrophin due to paralysis of the lymph hearts by

some substance contained in the urine (anesthetics, acids, urine preservers, toxics, etc.), a rare occurence, should be excluded (Valle, Penhos and Houssay, 1952). The beats of the lymph hearts are easily seen through the skin.

Time of reaction. — Exceptionally a positive test can be obtained in 10 (Galli Mainini, 1947) or 15 minutes (Houssay, 1947). The time of appearance or spermatozoa in the urine has been studied by Galli Mainini (1947, 1948), Blanchard and Bretto (1947); Sala *et al.* (1947); Sammartino and Arrighi (1948). At 1 hour, 50-55 % of the positive results are detected; at 2hs. 94.5 % (Galli Mainini, 1947, 1948); at 3 hs. practically 100%. Nevertheless, some positive reactions are more tardy and according to Sammartino and Arrighi (1948) about 1.5% of the positive results are detected only after 24 hs.

Persistence. — Spermatozoa disappear rapidly from the urine after a positive test. Generally, the reaction is already negative after 2 days (Merchante, 1947; Sammartino and Arrighi, 1948). Nevertheless, if was still positive once after 7, 8 and 9 days (Pou de Santiago, 1948). Persistence was higher at low temperatures. To prevent errors the absence of spermatozoa in the urine should be verified before the test. Histologically the spermating action disappears 92 hs after injection of chorionic gonadotrophin and 114 hs. after injection of seric gonadotrophin (Mancini and Burgos, 1947).

Serum or plasma. — The reaction can be obtained with blood, plasma or serum (Galli Mainini, 1947, 1948; Hutz, 1947; Gori, 1947; Schweitzer, 1951, etc.). Between 50 and 70 days of pregnancy, when gonadotrophin concentration is higher, a similar concentration is found in serum (or plasma) and urine; but in the following months the concentration in serum is higher than in the urine (A. Houssay, unpublished). Renal clearance of gonadotrophins is proximal to unity during the period of maximal concentration and diminishes to 0.3 to 0.4 in the following months (A. Houssay).

The toad is 3 times more sensitive to pregnant women's serum gonadotrophin than to the same extracted from serum. Spermiation is obtained in 2/3 of toads (1 *Bufo arenarum* unit) with 12 I U of pregnant women's serum and with 50 I U of serum extracted gonadotrophins. International units were determined in the rat using the ovary weight test (Schweitzer, 1951). It has been suggested that plasma contains something which inhibits the reaction in the rat or enhances the reaction in the toad.

Specificity. — In *Bufo arenarum* only pituitary (pituitary glands, F. S. H., L. H.) or chorionic gonadotrophins (pregnant women's urine, pregnant mare's serum, placenta, from women or mares) produce spermiation (1). Human urine only gives positive tests when coming from pregnant women or in cases of mole or *chorio-epithelioma.* We have no experience with cases of testicular *chorio-epithelioma* but in other batra-

(1) Recently Tabarelli Neto has obtained some cases of spermiation by sodium oxalate. The fact was conformed by Penhos in 3/20 *Bufo arenarum* (unpublished data).

chians spermiation was obtained in these cases. A positive reaction is never obtained with urine from women or men of different ages, castrated of not, or in menopause or affected with various diseases.

Conditions depending on the animal

Weight. — Male toads weighing 80 to 120 g should be used. Toads weighing less than 70 g are often immature. Large toads (180 to 260 g) are less sensitive.

Conservation. — Toads need humidity, but they do not live in water. When tested it is better to put them into a pitcher, with water covering part of their body, in order to obtain a good diuresis.

Captivity. — It is better to use toads in good general condition; nevertheless the reaction can be obtained in non fed toads, kept in captivity several months (Galli Mainini, 1948). As long term inanition produces regressive testicular alterations, if the toads are to be kept in captivity during months, it is convenient to feed them by introducing into the mouth (with forceps or digitally) 2 g pieces of calf liver or raw meat or worms, once or twice a week (Penhos and Cardeza, 1950, 1951).

Repetition. — A week interval should be allowed before repeating the test in a given toad. Although even 12 tests have been performed in the same toad, it is better to use it no more than 4 times (Galli Mainini, 1947; Hutz, 1947). Hutz injected once a week 100 I U of gonadotrophin into the same toad; after the 8th injection the reaction was poorer and it failed completely after the 10th. Nevertheless, after a month's rest the toad may be used again.

Temperature. — At low temperatures (4-10° C), the action of gonadotrophins is slower and a lesser proportion of positive results are obtained. It is best to maintain the animals at a temperature of 20-23° since the previous day and specially during the test. A positive reaction is obtained with the toads in a hot box at 35° (Fal and Trilla, 1949, 1950), or 50° (Hutz, 1947). As already stated the rate of the reaction increases with temperature.

Humidity. — Humidity favours urine formation which is necessary for obtaining a positive reaction.

Ilumination. — With threshold doses better results seem to be obtained in darkness (Galli Mainini, 1947, 1948) (fig. 15).

Toxicity. — Death of the toads due to the toxicity of the urine is infrequent: 1.1% (Galli Mainini, 1947, 1948); 1.2% (Gori, 1947-1950); 1.5 % (Blanchard and Bretto, 1947), less frequent than the death of rabbits in the Friedman test.

Other species. — The reaction has been obtained in at least 22 species of toads: *Bufo arenarum, B. americanus, B. bankorensis, B. bufo, B. calamita, B. cognatus, B. crucifer, B. D'Orbigny, B. ictericus, B. marinus, B. melanostictus, B. palmarum, vulgaris, B. paracnemis, B. regularis, B. spinulosus, B. stomaticus, B. terrestris, B. valliceps, B. variabilis, B. viridis, B. vulgaris, B. woodhousii.* In all these the reaction is

specific. In some, smaller amounts of urine or chorionic gonadotrophins may be used.

The reaction has been obtained in more than 28 species of other batrachians such as: *Caliptocephalus gayi, Cystignatus bibronii, Discoglosus pictus, Hyla annectens, Hyla cinerea, Kaloula pulchra, Leptodactylus ocellatus, L. pentadactyla, Microhyla carolinensis, Odontophrinus cultripe, Pelobates cultripes, Scaphiophus holbrookii, Xenopus laevis, Rana arvalis, R. catesbiana, R. dalmantina, R. esculenta, R. graeca, R. hexadactyla, R. limnocharis, R. narina, R. pipiens, R. plancyi, R. ridibunda,, R. temporaria, R. tigerina, R. vittigera.* For references see Bhaduri (1951) and Knepton (1951).

Some of these species react with smaller amounts of gonadotrophin than *Bufo arenarum.* In some the reaction cannot be elicited in some seasons of the year or the sensitivity is very low. In various species spermatozoa can be observed spontaneously in normal urine; thus causing false positive reactions (i. e. Xenopus). In each country the appropriate batrachian should be selected taking into account its sensitivity, easy obtainance and specificity of the reaction.

Factors depending on the gonadotrophin

Concentration. — In the paragraphs on "Dosis" and "Seasonal variations" we have mentioned the amount of gonadotrophins which are active on the toad. Aproximate quantitative determinations have been made in pregnant women's urine (Galli Mainini, 1497, 1948; Gori, 1947, 1950; Houssay, 1947). In some cases positive reactions have been obtained with as little as 0,2 ml (Gori, 1950); 0.1 and even 0.05 ml of urine during the period of maximal concentration between the 43rd. and 86th. days of pregnancy (Allende and Orias, 1951; Houssay, 1951 a). This corresponds to a concentration of 450.000 to 900.000 I U in 24 hs.

Quantitative estimation. — Determination of the concentration of chorionic gonadotrophin in pregnancy urine in international units has been made in *Bufo arenarum* Hensel (Galli Manini, 1947, 1948, Houssay, 1947; Gori, 1950) Studies on this subject are being carried out by A. Houssay.

Conservation. — The reaction has been obtained with urines conserved for 5 (Fal and Trilla, 1949, 1950); 7 (Gori, 1947) and even 10 days (Galli Mainini, 1947, 1948). Part of the activity is sometimes lost (about 50% in 10 days).

Time of pregnancy. — The most early positive reactions have been obtained 2 (Del Campo, 1951); 3 and 5 (Sammartino and Arrighi, 1948); and 4 to 5 days (Galli Mainini, 1947) after the missing menstrual period.

In the very recent cases the proportion of positive reactions is rather smaller (90% according to Ferrari et al, 1947). The proportion increases if the gonadotrophins are concentrated by adding 4 vol. of alcohol to 50 - 100 ml of acidified urine with addition of NaCl 0.1% (Del Campo de Hachen, 1951). In dubious cases the test should be repeated one or two weeks later, when gonadotrophin concentration increases.

TABLE V

Positive results of the Galli-Mainini reaction in Bufo arenarum Hensel. Pregnancies of less than 4 ½ months.

	Observations	Per cent positives
Galli Mainini	2027	99.01
Blanchard y Bretto	86	98.83
Blanchard, Viale y Pepa	639	99.70
Fal y Trilla	120	100
Ferrari, Pastori y Ledesma (*)	27	96
Figueroa Casas, Belizan y Staffieri	100	99
Gandolfo Herrera y Sauri	69	100
Gori	103	99.02
Haines	38	97.30
Heredia	45	95.50
Hutz	86	100
Merchante	100	98
Pinto y Suer Boero	82	100
Pou de Santiago	172	98.85
Rodríguez López	300	100
Sala, Jochesky y Colotta	50	98
Sammartino y Arrighi	409	97.10

(*) In 38 very recent pregnancies 90% positives were obtained.

In pregnancy of less than 4 1/2 months and especially between the 45th, and 70th days the proportion of positive results is very high (98 to 100%) (fig. 17). The animals should be in good condition, at an adequate temperature and 2, or even better 3, toads should be injected (Pinto and Suer Buero, 1947) in order to avoid negative results. The test is positive even if only 1 of the 3 toads react. The object of this test is the early diagnosis of pregnancy; in advanced pregnancy physical examination suffices and biological tests are superfluous.

The concentration of gonadotrophins in urine diminish during the third month. Pinto and Suer Buero (1947) obtained positive reactions in 100% of cases up to the 5th. month, 85.7% during the 6th., 83.3% during the 7th., 93,3% during the 8th., 90% during the 9th., 50% during delivery and 12.5% during puerperium. Between the 6th, to the 9th. month the proportion of positive tests diminishes to 92% (Galli Mainini, 1948); 93% (Merchante, 1947); 91% (Ferrari et al., 1947); 92% (Gori, 1947); 90% (Pou de Santiago, 1947, 1948); 92.5% (Heredia, 1948). The only divergent results are those of Gandolfo Herrera and Sauri (1947), who obtained 67% and of Hutz (1949) with 100%.

During puerperium the activity of the urine diminishes rapidly. Only 50% of positive results were obtained after 24 hs. (Pou de Santiago, 1947, 1948). In a few cases positive results were obtained 48 hs. post partum, but none on the third day (Pou de Santiago, 1947, 1948; Fal and Trilla, 1950).

Comparative sensitivity. — Galli Mainini's test has been compared to Friedman's test by various workers (Galli Mainini, 1948). The toad has proved slightly less sensitive to chorionic gonadotrophin than the female rabbit (Houssay. 1949 a, b; Cheymol et al, 1952). In 1.000 test with pregnant women's urine Friedman's test failed 3 times (3.0%) and Galli

Mainini's test 12 times (1.2%) (Sammartino and Arrighi, 1948). In 639 cases with positive Friedman tests Galli Mainini test was negative 3 times (Blanchard, Viale and Pepa, 1950). The female rabbit is also slightly more sensitive than the european *Bufo bufo* (Thorborg, 1951). In shorter series results are variable (Pinto and Suer Buero, 1947, etc.).

Various clinical conditions. — A positive Galli Mainini's test indicates the presence of an implanted living placenta which secretes gonadotrophins.

The test is positive in many cases of mole and becomes negative when it is eliminated. (Pou de Santiago, 1947, 1948; Pinto and Suer Buero, 1947; Galli Mainini, 1948; Heredia, 1948, etc.). It has been found markedly positive in cases of chorio-epitheliona (Allende and Orías, 1950, 1951).

It is also positive in many, but not in all, cases of ectopic pregnancy (Pinto and Suer Buero, 1947; Merchante, 1947; Pou de Santiago, 1947; Hutz, 1947), etc.). 2 of 7 cases (Galli Mainini, 1948), 8 of 15 cases in which 11 were Friedman's positive (Sammartino and Arrighi, 1948).

In impending or incomplete abortion a positive reaction may occur in many cases, though it is negative in others (Galli Mainini, 1947, 1948; Pinto and Suer Buero, 1947; Blanchard and Bretto, 1947; Pou de Santiago, 1947-48, Hutz, 1947, etc.). Friedman's and Galli Mainini's tests were parallel in all but 3 cases in which the former only was positive.

It has been found out that Galli Mainini's test may fail in two pathological conditions where the clinician needs urgent and precise information i.e. ectopic pregnancy and impending abortion (Sammartino and Arrighi, 1948).

The test may be negative in a) very recent pregnancies, because the placenta does not secrete yet enough gonadotrophins. One or two weeks later, the test, if repeated, will yield positive result; b) in ectopic pregnancy, impending or incomplete abortion, because the placenta secrets less gonadotrophin or has ceased to secrete it. In these cases the following procedure should be adopted: 1) perform a Galli Mainini's test; 2) if after 2-3 hs. the test is negative two female rabbits are injected and the result verified after 24hs. or else the urine is concentrated (Del Campo de Hachen, 1951) and a new Galli Mainini test performed. The injection of 5 ml of the woman's serum into the toad can also be tried because serum is more reliable than urine.

Advantages. — The principal advantages of Galli Mainini's test for the early diagnosis of pregnancy are:

1) *Accuracy.* — Since it is positive in 98-99% of pregnancies of less than 4 months and is always negative in non pregnant women except in cases of mole or chorio-epithelioma.

2) *Reliability*: since positive reaction always indicates pregnancy, except in the mentioned cases of moles or chorioepithelioma.

3) *Specificity*: since no other substance apart from pituitary or placental gonadotrophin elicits a positive reaction.

4) *Celerity*: since a response is obtained in 1 - 3 hs.

5) *Easiness of technique*: since the procedure is rapid and simple, the urine has not to be prepared, nor the toads operated upon, the final

reading is easy, no coloration nor fixation being necessary and the error is practically impossible.

6) *Precocity*: because it diagnoses pregnancies of very little more than one month and is very accurate between 45 and 70 days of pregnancy.

7) *Sufficient sensitivity*: which can, if necessary, be increased by concentrating the urine or injecting the serum.

8) *Economy*: the animal being cheap and may be used for several tests without being fed. These merits have been analyzed by Galli Mainini (1948).

World diffusion. — Galli Mainini's test in ***Bufo arenarum*** is used in Argentina, Uruguay, southern Brasil and southern Bolivia. Employing the batrachians prevalent in each country it has been practised in many countries of every continent. Publications of 40 countries are known to us: Argentina, Australia, Austria, Belgium, Birmania, Bolivia, Brasil, Canada, Ceylan, Chile, China, Colombia, Finland, France, Germany, Golden Coast, Great Britain, Holland, Hong Kong, Hungary, India, Ireland, Israel, Italy, Japan, Mauritius, Mexico, New Zealand, Nicaragua, Palestine, Paraguay, Peru, Poland, Russia, Sweden, Switzerland, United States, Uruguay and Venezuela.

Diagnosis of pregnancy in domestic animals

Mare. — The toad is more sensitive to chorionic gonadotrophin of pregnant women's urine than to the gonadotrophin of pregnant mare's serum. To obtain spermiation in 2/3 of toads (one ***Bufo arenarum*** unit of Schweitzer and Bas) 38 I U (rat) of chorionic gonadotrophin and 150 I U (rat) of seric gonadotrophin are needed. A toad unit is equivalent to 28 I U of mare's hypophysis and 17 I U of horse's hypophysis (Schweitzer and Bas, 1948, b, c; Schweitzer, 1951 b) by titration in rat.

Nevertheless, the toad is useful for 1) early diagnosis of pregnancy in the mare; 2) deciding the best period for bleeding the mares in order to prepare gonadotrophin for therapeutic uses in humans; 3) diagnosis of twin pregnancy.

Twelve ml of mare's serum should be injected to each toad (one or two). The urine is examined after 3, 6, 22 and 24 hs. for the presence of spermatozoa. The test has always been negative in the absence of pregnancy. It was found positive in the serum of 33 out 37 mares with pregnancies of 48 to 58 days (88.9% of cases). Adding 69 observation in pregnant or non pregnant mares, the test gave a correct answer in 94% of cases (Schweitzer and Bas, 1948 a; 1949). Only 2.6% of the injected toads die.

The study of hundreds of animals has shown that each mare has an individual gonadotrophin concentration curve, which occurs between the 40th. and 100th. day of pregnancy with a maximum between the 60th. and 70th. In the following pregnancies the curve repeats itself with similar characteristics (Schweitzer, 1949).

Twin pregnancy, which is rare in horses, is accompanied by a higher gonadotrophin concentration (nearly double) than that which the same mare showed in previous single pregnancies. This is due, apparently, to

the fact that in twin pregnancy placentary tissue which secretes gonadotrophin is approximatedly doubled (Schweitzer, 1949 b).

Pregnant mare's urine does not produce spermiation in the toad.

Tabarelli Neto (1949) performed this test injecting subcutaneously to each toad 9 to 20 ml of pregnant mare's serum. The toads used were *Bufo marinus* and *Bufo paracnemis* and the pregnancies of 36 to 111 days. At the same time female rabbits were injected (modified Cole-Hart's test). In 39 tests results were coincident in both animals: 25 positive and 11 negative. In 3 tests results were divergent: in 1 case the test was positive in the toad and negative in the rabbit, when repeated it gave positive result in both; in the other 2 cases the reaction was negative in toads and positive in the rabbit; when repeated it became positive also in one of the the toads tests. On the whole, the toad gave 92% of positive results in pregnant mares.

More recently Tabarelli Neto (1953) injecting 15 ml of mare's serum into *Bufo marinus* or *B. paracnemis* found 97.7 % of positive reactions in 15 pregnant mare's and 93.9% in another series of 101 pregnant mares in which the Friedman test was positive in 99.4 % of the cases. The optimum time is between 45 and 94 days. A positive test can be obtained until 25 days after abortion.

Ruminants

Serum and urine of all the domestic species studied have not been able to produce spermiation in the toad (see "Spermiation"). The serum of other equinus than the mares has not yet been studied.

Bhaduri and Bardan confirmed this with urine from cows and other domestic animals. Later they macerated feces of pregnant cows (15 g in 100 ml water) centrifuged and filtered. The resultant fluid was injected into the toad *Bufo melanostictus* 5 ml every hour until the 4 th. hour. Spermiation was obtained in 100 % of cases of pregnancy in cows and buffalo, and positive results were obtained also in ewes, female stags and antilopes. In goats only in rare occasions (Bhaduri and Barden, 1949, 1950; Bhaduri, 1950, 1951).

Many essays in *Bufo arenarum* Hensel were always negative (Nelson, Houssay, Galli Mainini). In Spain, Aznar - Ferreres (1951) obtained positive reactions injecting dialized cow's feces into *Rana ridibunda perezi*. The active substance was water soluble, insoluble in ether and precipitated by alcohol. But positive tests were obtained with feces from pregnant or non pregnant cows, which discards the use of this test for the diagnosis of bovine pregnancy.

Tabarelli Neto (1954) has obtained few inconstant reactions in *Bufo marinus* and *B. paracnemis*, using feces of pregnant cows. He mentions that Noguera has obtained some positive results in *Bufo marinus* with extract of feces of pregnant and non pregnant cows and of bulls.

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NEPHROGENIC DIABETES INSIPIDUS INDUCED BY AMINOPTERIN

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RATS treated with Aminopterin (4-aminopteroylglutamic acid), at a dosage of 5 μg per 100 g of body weight daily, show towards the end of the intoxication period an increase of water intake and of urine output which is not associated either with an increase of NaCl intake or with the presence of glucose in the urine. This condition resembles, therefore, a diabetes insipidus and thus it has been considered (Lewis et al., 1952; Rabasa et al., 1953).

The present experiment has been undertaken as an attempt to elucidate the cause of this change. Two hypothesis have been considered: a) a decrease of the production and/or release of antidiuretic hormone by the pars nervosa of the hypophysis, and b) a lack of sensitivity of the renal tubuli where the hormone exerts its effect. To check the first hypothesis it was decided to assay the antidiuretic activity of serum of rats with diabetes insipidus due to Aminopterin intoxication (D. I.) comparing it with normals. For the second, normal rats and rats with D.I. were treated with tannate of Pitressin to ascertain their sensitivity to antidiuretic hormone.

In preliminary experiments (Rabasa and Cortés, 1953) it was shown that the antidiuretic activity of the serum of rats with D. I. did not depart significantly from that of normals. The assays were made with the method of Birnie et al. (1949) slightly modified. This part was repeated in the present experiment with the method of Ginsburg and Heller (1953) because of its greater sensitivity.

METHOD

Female rats with an average weight of about 150 g were injected with Aminopterin * at a dosage of 5 μg per 100 g of body weight daily.

* The authors are indebted to Dr. J. M. Ruegsegger, from the American Cyanamid Co. for the Aminopterin.

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When the diabetes insipidus was obtained they were distributed in four groups of 5 rats each. Ten normal female rats of the same age and weight were added to each group as controls.

To see the effect of Pitressin all the rats were hydrated by gastric tube with a water load of 9% of their body weight, administered in three doses of 3% each, separated by an interval of 30 m first and 60 m afterwards. One of the groups received 1 I U of tannate of Pitressin * after the last dose of water was administered. Another group received 1 I U 2 h 30 m before hydration. A third one received 0.5 I U 3 h 30 m also before hydration and a fourth one remained as uninjected control. A half of the controls received Pitressin at the same dosage and at the same time as the rats of their group; the other half remained uninjected.

The rats of one of the groups receiving 0.5 I U were hydrated one day and injected with Pitressin and hydrated again the following day, being thus taken as their own uninjected controls (Figs. 1 and 2). The urine excretion was recorded during the hydration period (90 m) and 3, 6 and 18 h later.

The assay of the antidiuretic activity of serum and of the posterior pituitary lobe was performed by the method of Ginsburg and Heller (1953), using male rats with an average weight of 200 g. A polithene tube was inserted in the left external jugular vein, coming out through the skin between the ears. The end was closed with a removable cap. The substance to be assayed was injected intravenously through this tube. The quantity was always 1 ml followed by 0.5 ml of the heparin solution. It was taken as standard 1/1000 of one posterior pituitary of rat, which roughly represents 300 μU of antidiuretic hormone. No assay of an unknown sample was started unless two conditions were fulfilled, namely: a) lack of antidiuretic response to 1 ml of a solution of 1/10.000 of heparin in normal saline, and b) a good response to 1/1.000 of one posterior lobe of the pituitary of rat.

To calculate the antidiuretic effect the following method was used: it was taken as normal level of excretion the mean of the previous and following records which limit the period of oliguria. The antidiuretic activity was measured by the difference between the quantity actually excreted and the expected amount had no antidiuretic substance been injected. This is expressed as ml and is called deficit of excretion. When there is an antidiuretic activity it is, obviously, negative.

The serum was obtained from the heart or the external jugular vein of rats under ether anesthesia.

RESULTS

Antidiuretic activity of serum and posterior pituitary of rats with D. I.

The intravenous injection of 1 ml of the heparin solution has no antidiuretic effect (Table I). One ml of serum either from a normal rat

* The tannate of Pitressin was obtained through the courtesy of Dr. Hipólito González, from Parke Davis and Co.

TABLE I

Deficit of excretion induced by posterior pituitary extracts and serum of normal rats and rats with D. I.

Substance		Title	Effect (Def. of Excret.)	n
Posterior pituitary	Normal	0.01	— 4.80	1
		0.001	— 2.29	19
		0.0005	— 2.30	3
		0.00025	— 1.10	1
	D.I.	0.001	— 1.83	4
Heparin		0.0001	0.09	33
Serum	Normal	1	— 2.10	7
	D.I.	1	— 2.03	5

or from a rat with D.I. showed slightly less antidiuretic activity than 1/1.000 of one posterior pituitary of rat, that is about 300 μU of antidiuretic hormone. Extracts obtained from the pituitaries of two rats with D. I., assayed two times each, had the same potency as the normal ones.

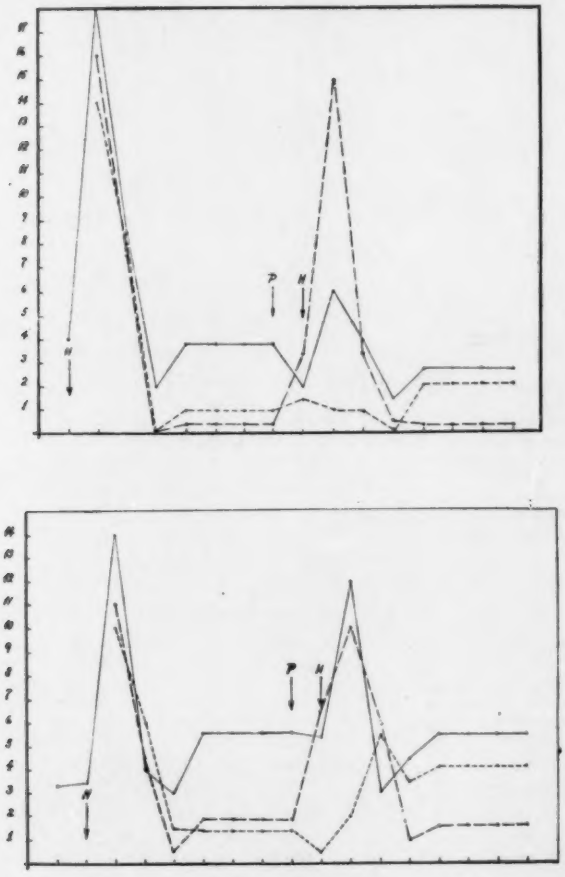
Effect of hydration. — Rats with D.I. excrete more urine than normals after the experimental hydration ($p<0.05$) (Figs. 1 and 2). Four and a half hours after the start of hydration, both normals and rats with D.I. showed an inhibition of their urine excretion which is longer in the former. The proportion of this inhibition is of the same order in both cases, no urine excretion being recorded during the entire length of this period sometimes (even in two rats out of six with D.I.). When it ceased the urine output reached approximately its previous level.

Effect of Pitressin. — When 1 I. U of tannate of Pitressin was injected at the completion of hydration its effect and the spontaneous inhibition just described confounded themselves. When the same dose was injected 3 h 30 m before hydration both rats with D.I and normals showed an inhibition of their urine excretion during the hydration period and although the level of the rats with D.I. remained higher the difference was not significant (Table II).

During the following six hours, however, the rats with D.I. injected with Pitressin showed no more inhibition while their normal controls also injected with Pitressin remained with a low excretion. This difference is significant at the level of 0.05.

Tannate of Pitressin at a dosage of 0.5 I. U injected 3 h 30 m before

hydration induced, during the hydration period, an inhibition which is significantly milder in rats with D. I. than in normals (See Figs. 1 and 2 and Table II). It can be seen besides (Table II) that while no difference of response exists in normals between the doses of 1 and 0.5 I. U of tannate of Pitressin, a significant difference is seen with these two doses in rats with D.I.



FIGS. 1 and 2. — *Hydration with and without Pitressin in rats with D. I. and controls. Full line: Rat with D. I. Dotted line: Normal rat with Pitressin. Broken line: Normal control without Pitressin in both stages. Abscissa: Time in periods of 3 h. Ordinate: Excretion of urine in ml H: Hydration P: 0.5 I. U. of tannate of Pitressin. These two graphs show the range of sensitivity of rats with D. I. to Pitressin.*

TABLE II

Urine excretion of rats with D. I. and normals treated with tannate of Pitressin

Periods of excretion	D.I.			Normals		
	Pitressin		Controls	Pitressin		Controls
	0.5 I.U	1 I.U		0.5 I.U	1 I.U	
Hydration period	4.03	2.10	7.06	0.50	0.81	4.50
Six hours later	5.98	8.87	8.01	3.83	3.54	6.70

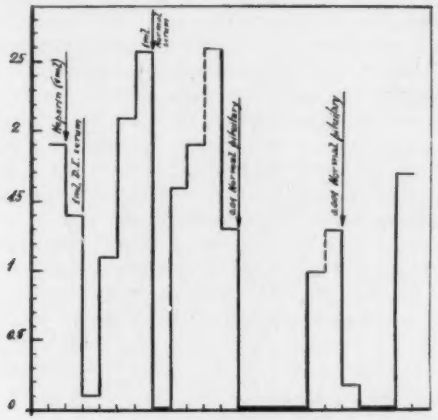
Analysis of Covariance (data of Table II).

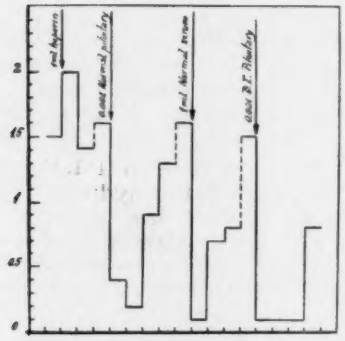
Sources		D. of F.	Sum of squares	Mean square	F.
Hydration	**period**				
	Pitressin	1	71.15		60.30
0.5 I.U.	D.I.	1	34.04		28.85
Pitressin	Interaction	1	3.75		3.18
	Error	15	17.73	1.18	
	Pitressin	1	82.14		85.56
1 I.U.	D.I.	1	25.11		26.16
Pitressin	Interaction	1	3.09		3.22
	Error	15	14.45	0.96	
	D.I	1	27.48		26.42
Dosage	Dose	1	3.88		3.74
	Interaction	1	7.65		7.36
	Error	15	15.63	1.04	
Six hours	**later**				
	Pitressin	1	18.37		2.39
0.5 I.U.	D.I.	1	27.05		3.51
Pitressin	Interaction	1	0.16		0.02
	Error	15	115.50	7.70	
	Pitressin	1	19.47		2.49
1 I.U.	D.I.	1	30.94		3.96
Pitressin	Interaction	1	34.69		4.44
	Error	15	117.32	7.82	

(F 0.05 = 4.54; F 0.01 = 8.68)

Discussion

The antidiuretic activity of serum cannot be considered with certainty as a test of the presence of antidiuretic hormone of pituitary origin, although increasing evidence is being accumulated in favour of this view. There is no such activity in the serum of hypophysectomized rats (Birnie et al, 1950; Ames and van Dyke, 1952). Its activity is greater in the jugular vein than in the carotid (Ginsburg and Heller, 1953). Alcohol anesthesia inhibits it while ether anesthesia increases it (Ames and van Dyke, 1952; Dicker, 1953). It is not present in the blood of





FIGS. 3 and 4. — *Tests of antidiuretic activity of posterior pituitary lobe and serum of rats with D. I. and normals. (Method of Ginsburg and Heller). Abscissa: Time in periods of 10 m. Ordinate: Excretion of urine in ml.*

rats decapitated without any previous stimulation (ether anesthesia, pain, fear, etc.) which could cause the release of the hormone by the pituitary (Ames and van Dyke, 1952). Unpublished experiments in this laboratory show besides that it dialyzes through the same membrane as antidiuretic hormone, it is adsorbed in the same column of permutit and is eluted by the same NaCl solution. It can be concluded therefore that the presence in the serum of rats with D.I. of an antidiuretic activity similar to that of normals favours, although it does not prove, the hypothesis that the production and release of antidiuretic hormone by the posterior lobe of the hypophysis is approximately normal. The hypophysis itself contained moreover a normal quantity of antidiuretic hormo-

ne in the two rats assayed although the urine excretion and water intake was severalfold that of normals.

It is commonly assumed that hydration inhibits the release of antidiuretic hormone, but it has been observed (Newton, 1949) and confirmed in this laboratory that even with water loads of 6% of body weight an inhibition of urine excretion can be brought about by the operation of hydrating the rats by stomach tube. This inhibition is immediate and very short, it lasts only about 20 m and it has been observed also in a rat with D.I. hydrated in the same conditions.

Normals and rats with D.I. show besides 4 h and 30 m after the start of hydration another inhibition of urine excretion which incidentally may lead to a complete anuria during three or more hours. The rats with D.I. returned rapidly to their high level of excretion while normals remained a longer period without any excretion or with a very low one. There is no easy explanation for this fact.

If the delayed inhibition was also due to the effect of the antidiuretic hormone its release should have been produced by the stimulus of hydration 4 h 30 m before and the delay of this action might be attributed to the threshold of the target organ, which, contrary to the current opinion (Dicker, 1953), shoud be supposed to decrease together with the water load. Thus two kinds of inhibition due to antidiuretic hormone could perhaps be produced: one immediate of very short duration and several hours later a longer one which might be only possible when the water load be lower. If this interpretation proved correct, it should mean that the rats with D.I. are capable of an antidiuretic response when properly stimulated.

When these rats are injected with 1 I.U. of tannate of Pitressin the inhibition they show during the hydration period does not depart significantly from that of normal injected ones, but in the following six hours a return to their high excretion is seen while normals remain inhibited. When 0.5 I.U. are given the response during the hydration period is significantly milder than that of normals, the latter showing no difference with the two doses. It seems safe to conclude that the threshold of normals to the antidiuretic hormone is lower than that of rats with D.I. and a full response can be brought about with 0.5 I.U. while in rats with D.I. is not yet complete even with 1 I.U. Two dose-response curves significantly different can be obtained if the sensitivity of normals to the antidiuretic hormone is compared to that of rats with D.I.

The available experimental data points therefore towards the existence in the kidney of a system (enzymatic ?) which needs folic acid or its derivatives to respond adequately to the antidiuretic hormone and which can be inhibited, consequently, with an antifolic (Aminopterin). No complete exhaustion of it has been achieved since the rats with this condition respond to higher doses of antidiuretic hormone.

The D.I. due to Aminopterin is reversible since some of the rats with D.I. survive and their urine excretion returns then to its normal level.

As far as the authors are aware no other nephrogenic experimental diabetes insipidus is known, but in human pathology an entity thus ca-

lled exists (Williams and Henry, 1947; Yun-Chen Kao and Steiner, 1953) which is also due to a lack of sensitivity of the renal tubuli to the antidiuretic hormone. The present experimental approach could prove useful to disclose the origin and mechanism of this condition and could also help to find out the site of action of the antidiuretic hormone in the kidney.

Summary

Rats injected with low doses of Aminopterin show a diabetes insipidus (D.I.).

The serum and posterior pituitaries of these rats have the same antidiuretic activity as normals.

Rats with D.I. apparently may release antidiuretic hormone when stimulated by hydration with stomach tube.

The renal threshold to the antidiuretic hormone is significantly higher in rats with D.I. This D.I. is considered, accordingly, as a nephrogenic one.

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ACTION DE DIVERS CURARISANTS SUR L'ORGANE ELECTRIQUE DE L'ELECTROPHORUS ELECTRICUS L.

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COMPARER le fonctionnement des effecteurs périphériques que sont les organes électriques de certains poissons à celui d'autres effecteurs a préoccupé depuis longtemps les physiologistes. Par suite de leur origine embryologique commune, on a le plus souvent rapproché ces organes des muscles striés; mais les résultats de leur stimulation électrique directe ont été si ambigus que l'on pouvait encore récemment se poser la question de savoir si leurs propriétés étaint assimilables à celles de l'effecteur musculaire proprement dit, ou si on devait les considérer plutôt comme analogues à la plaque motrice, ou encore à une différenciation particulière des éléments nerveux terminaux.

Devant ces difficultés, les physiologistes ont depuis longtemps essayé d'employer les méthodes pharmacologiques qui avaient déjà servi, dans l'étude du muscle, à dissocier la part des éléments nerveux de celle des éléments musculaires proprement dits. C'est à Gotch (1888), Gotch et Burch (1896) et Schönlein (1896) que sont dus les premiers essais corrects de curarisation de la jonction nerf-électroplaxe; ces premières études portèrent sur les organes de la Torpille, de la Raie et du Malaptérure.

Employant du curare naturel à diverses doses (jusqu'à 40 cg par kg par voie artérielle chez la Torpille), ces auteurs montrèrent que ce produit était inefficace sur la transmission nerf-électroplaxe, bien qu'il ait bloqué chez les mêmes animaux la transmission neuro-musculaire.

Auger et Fessard, en 1932, essayèrent à nouveau et sans plus de succès la curarisation de l'organe de Torpille. Devant l'inefficacité des injections ils utilisèrent ultérieurement (1941) l'immersion de fragments de tissu dans des solutions de curare. Ce n'est qu'à condition d'employer

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des prismes isolés, légèrement incisés afin de favoriser la pénétration du produit, qu'ils obtinrent la curarisation après environ une heure d'immersion dans des solutions contenant 1 p. 100 de curare naturel. Signalons enfin que des contrôles récents faits par l'un de nous (non publiés) ont montré que l'on pouvait obtenir la curarisation totale de fragments identiques après une demi-heure d'immersion dans des solutions contenant 0,03 p 100 de *d*-tubocurarine.

Cependant, récemment encore Chagas et Bovet (1953), reprenant les expériences de curarisation chez la Torpille intacte, ont montré que 20 mg de Flaxédil ou de *d*-tubocurarine par kg d'animal, injectés par voie intra-abdominale étaient inefficaces sur la jonction nerf-plaque.

L'ensemble de ces résultats montre donc, ainsi que l'avait admis Gotch (Traité de Schäfer, 1898) que la structure acceptrice de la jonction nerf-organe chez ces Poissons ne se comporte pas vis-à-vis des curarisants à molécule complexe de la même façon que celle de la jonction nerf-muscle strié, encore que cette dernière présente d'assez grandes variations de susceptibilité selon l'espèce et le type de muscle.

Il importe maintenant de rappeler que dans les trois poissons électriques chez lesquels l'action des curarisants avait été jusque-la étudiée, aucune individualisation électrophysiologique n'a encore pu être obtenue de l'effecteur, considéré indépendamment des terminaisons d'axone qui l'innervent. Le fait que la stimulation prétendue directe de fragments d'organe est suivie chez les Torpilles comme chez les Raies de réponses à longues latences (4 ms au seuil, 1.5 ms pour la stimulation maximale chez la Torpille; 2.5 ms chez la Raie), identiques quelle que soit la direction du courant stimulant, semble prouver que cette stimulation doit toujours passer par les rameaux nerveux terminaux. Les expériences sur le Malaptérure qui n'ont malheureusement pu être reprises depuis Gotch (1896) parlent en faveur de la même hypothèse.

Les effecteurs de ces trois Poissons n'auraient donc pas de véritable excitabilité électrique directe comparable à celle d'un muscle strié. Au contraire, chez le Gymnote (Electrophorus electricus) depuis les expériences de Albe-Fessard, Chagas et Martins-Ferreira (1951, *a* et *b*) (complétées par une étude détaillée de Martins-Ferreira sur les effets de température) on sait qu'il existe un organe électrique effecteur directement excitable.

Dans ces expériences, des fragments d'organe (organe principal) placés entre deux plaques d'argent servant d'électrodes à la fois stimulatrices et receptrices, étaient soumis à des stimulations par des courants homodromes ou hétérodromes (1), Dans le sens hétérodrome deux réponses sont obtenues, l'une à très courte latence (ordre de 0.1 ms) qui est la réponse de l'électroplaxe excitée directement, l'autre se présentant 1.5 à 2 ms aprés la stimulation, nécessitant l'addition de plusieurs stimuli pour apparaître, se fatiguant beaucoup plus vite que la première et ayant toutes les caractéristiques de la réponse obtenue par l'intermédiaire d'un nerf électrique. Cette dernière réponse ne peut être due qu'à la stimulation

(1) Courants homodromes et hétérodromes: courants dirigés perpendiculairement aux électroplaxes, soit dans le sens de la force électromotrice (courants homodromes) soit en sens inverse (courants hétérodromes).

des rameaux nerveux terminaux qui se trouvent dans le bloc d'organe. Dans le sens hómodrome, une seule réponse apparaît qui a toutes les caractéristiques de la seconde réponse obtenue dans le sens hétérodrome. Ce fait nous confirme done que la première réponse obtenue avec un courant hétérodrome correspond à un élément à excitabilité directionnelle: il s'agit bien de la réponse directe de la plaque.

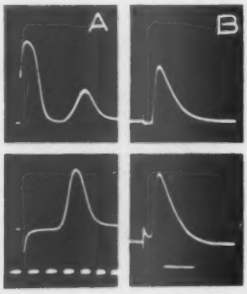

FIG. 1. — *Comparaison des décharges obtenues par stimulation de fragments d'organés électriques placés entre deux électrodes parallèles aux plans des électroplaxes: A, chez "Electrophorus"; B, chez "Raja clavata". Tracés supérieures, stimulation par courant hétérodrome. Tracés inférieures, stimulation par courant homodrome.*
En A, temps en ms, en B le trait correspond á 10 ms. Noter que seule le réponse de "l'Electrophorus á la stimulation hétérodrome comporte une premiére onde à très courte latence.

Ayant donc trouvé un organe électrique contenant un effecteur excitable, il était possible de reprendre l'étude de l'action des substances curarisantes sur la jonction nerf-organe électrique.

Dans des recherches préliminaires, Albe-Fessard et Chagas (1951) montrèrent une très nette différence de comportement entre les réponses directes et celles d'origine nerveuse, en perfusant des tronçons d'*Electrophorus* à l'aide de solutions de curarisants de synthèse, soit *d*-tubocurarine, soit chlorydrate de bébérine (fig. 2). La technique de perfusion utilisée était celle déjà décrite par Chagas et coll. (1951). De ces premiers résultats, il ressort que la *d*-tubocurarine amène une perte de la réponse indirecte (réponse d'origine nerveuse) sans altérer la réponse directe. Postérieurement Chagas, Bovet et Sollero (1953) étudièrent l'action des curares à molécules complexes sur l'*Electrophorus* conservé vivant hors de l'eau, grâce à une circulation buccale d'eau. Ils montrèrent que l'injection intracardiaque de *d*-tubocurarine ou de Flaxédil produit une rapide curarisation tant musculaire qu'électrique de l'animal; une dissociation se produit cependant entre le comportement des muscles et celui de l'organe envers ces agents: le muscle récupère totalement après six à huit heures pour des doses qui suppriment le fonctionnement de l'organe pendant 72 heures.

Il convenait ensuite d'étudier les actions comparées de divers curarisants, et en particulier d'opposer aux curarisants à molécules com-

plexes tels que Flaxédil ou *d*-tubocurarine, les curarisants du type ammonium quaternaire: c'est ce travail que nous présentons ici.

TECHNIQUE

Les expériences ont porté sur 21 poissons dont les poids étaint compris entre 1 et 7 kg environ (poids moyen approximatif: 4 kg). Deux méthodes furent utilisées pour introduire les solutions curarisantes:

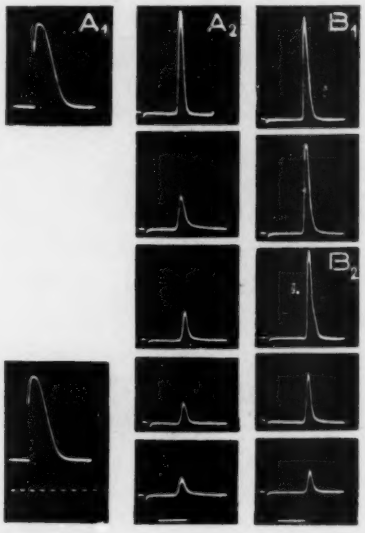

FIG. 2. — *Tracés obtenus au cours de la perfusion de deux tranches d'Electrophorus A1 et A2 - Perfusion par une solution de d-Tubocurarine (30 mg par litre de solution) dans du sérum physiologique. A1 - réponses á la stimulation directe: en haut, au début de la perfusion - en bas, aprés 30 min de perfusion. Temps en ms. A2 —réponses successives á la stimulation nerveuse, au cours de cette même perfusion.. B1 - Perfusion d'un fragment témoin par du sérum physiologique, á partir de B2 on commence á perfuser le fragment témoin par la solution curarisante. Temps, A1, B1, B2, le trait correspond á 10 ms.*

a. la perfusion de tranches d'organe par du sérum physiologique contenant la subtance (technique déjà mentionnée plus haut);

b. l'injection par voie intracardiaque ou intra-arterielle du poison, chez l'animal entier placé dans des conditions de respiration artificielle.

La décharge directe est recueillie dans les deux cas par deux électrodes de 1 cm² environ qui servent en même temps d'électrodes pour la stimulation directe. La décharge d'origine nerveuse est, soit une réponse réflexe obtenue par stimulation naturelle, soit une réponse à la stimulation simultanée de quelques nerfs électriques à leur sortie du canal spinal. Elle est recueillie en général par deux électrodes interceptant un grand segment d'organe (moitié antérieure ou totalité de l'organe prin-

cipal, selon les expériences). Les deux méthodes de stimulation nerveuse ont indifféremment donné les mêmes résultats. Nous avons ainsi pu en déduire que les poisons curarisants n'agissaient pas sur les centres aux doses que nous avions employées et que par conséquent il était légitime pour obtenir la décharge d'origine nerveuse d'utiliser la stimulation naturelle, non traumatisante.

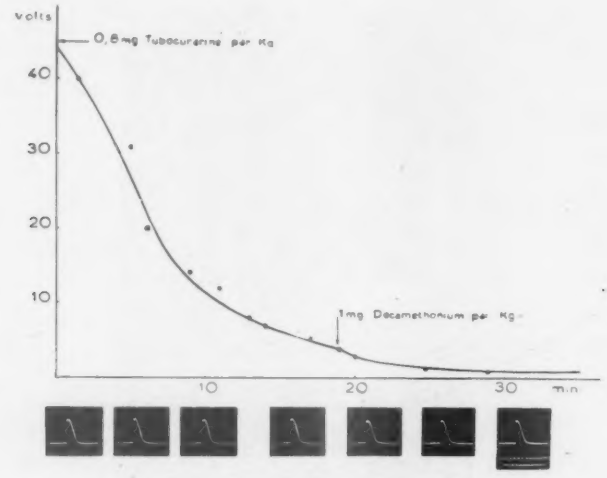

FIG. 3. — *Modifications de l'amplitude des décharges d'une partie de l'organe principal d'un "Electrophorus" à la suite d'une injection intracardiaque de 0,8 mg/Kg de d- tubocurarine, puis au bout de 20 min de 1 mg/Kg d'iodure de décaméthonium.*

La courbe montre l'évolution des tensions de la décharge obtenue par voie réflexe de la moitié antérieure du poissin. Les enregistrements alignés au bas de la courbe représentent aux instants correspondants, l'allure de la réponse directe recueillie sur le même poisson également dans la partie antérieure. Remarquer l'invariabilité de la réponse directe, même après l'injection d'iodure de décaméthonium).

Dans quelques expériences, nous avons de plus déterminé l'impédance du tissu électrique; nous utilisions de larges fragments d'organe laissés en place dans le poisson ou dans une tranche perfusée (prismes de 10 cm de long, 3 cm^2 de base). L'impédance équivalente de ces fragments était mesurée à l'aide d'un pont de Wien alimenté par un générateur à fréquence variable (les mesures furent faites dans les bandes de fréquence comprises entre 100 et 10.000 c/s.).

Les stimulations électriques étaient produites par un générateur d'impulsions à fréquence variable. Deux oscillographes étaient utilisés pour l'observation des décharges. Sur l'un des écrans étaient stabilisées et photographiées les réponses directes; sur l'autre apparaissaient les

réponses indirectes, dont on se contentait généralement de mesurer l'amplitude à intervalles réguliers.

RESULTATS

1°) *Action de la d-tubocurarine ou du Flaxédil*

Reprenant les expériences déjà citées de Albe-Fessard et Chagas (1951) et de Chagas, Bovet et Sollero (1953) nous avons confirmé leurs

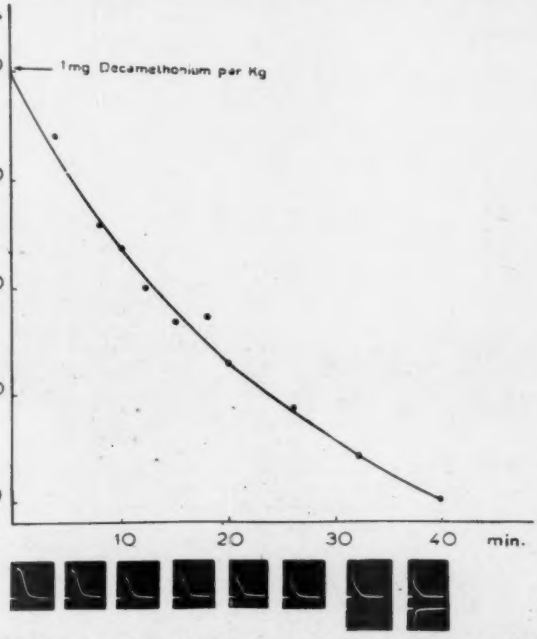

FIG. 4. — *Modifications de l'amplitude des décharges d'une partie de l'organe principal d'un "Electrophorus" á la suite de l'injection intracardiaque de 1 mg/Kg d'Iodure de décaméthonium.*
La courbe montre l'évolution des tensions de la décharge obtenue par voie réflexe de la moitié antérieure du poisson. Les enregistrements alignés au bas de la courbe représentant aux instants correspondants l'allure de la réponse directe recueillie sur le même poisson également dans la partie antérieure. Remarquer la décroissance parallèle des deux réponses. Le dernier tracé est accompagné du choc stimulateur appliqué dans le sens non efficace afin de montrer la forme de l'artefact pur.

résultats, à savoir que données en injection ou en perfusion, ces deux substances font baisser l'amplitude de la décharge d'origine nerveuse, jusqu'à totalment l'annuler, sans que la plaque électrique perde sa capacité de répondre à la stimulation directe (Fig. 2, 3, 7). Aucune modification appréciable de l'amplitude de la décharge directe, ou du seuil de stimulation, n'a été observée.

2°) *Actions de l'iodure de décaméthonium et de la succinylcholine*

Introduits en injection ou en perfusion, l'un ou l'autre produit provoque la baisse de la décharge d'origine nerveuse, et contrairement au cas précedent, provoque simultanément la baisse puis la disparition de la décharge directe. (Fig. 4, 5, 6, 7).

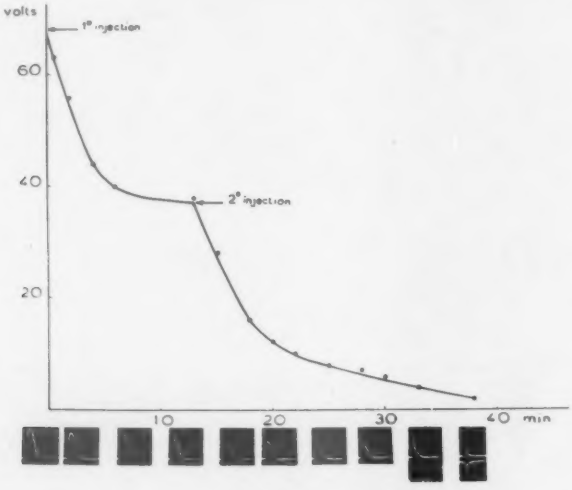

FIG. 5. — *Expérience analogue aux précédentes (3, 4). Action de deux injections succesives de 1 mg/Kg de succinylcoline: courbe, sur la réponse réflexe; enregistrements, sur la réponse directe.*

La fig. 4 présente une expérience dans laqueelle 4,7 mg de Décaméthonium avaient été injectés à un poisson de 4.7 kg. Les fig. 5 et 6 montrent pour deux injections de succinylcholine la décroissance d'amplitude des décharges d'origine nerveuse (courbe) et la modification de la forme des décharges directes (enregistrements). Enfin, pour la succinylcholine, l'action de doses croissantes a été étudiée; la fig. 6 montre l'effet d'injections successives de faibles quantités du produit.

L'injection préalable de sulfate d'ésérine à l'animal permet de supprimer la décharge directe avec une quantité de 100 μg/kg de succinylcholine injectée par voie cardiaque à un poisson de 4 kg. Nous avons en outre observé que de faibles doses d'iodure de décaméthonium peuvent avoir un effet initial de léger accroissement de l'amplitude (voir fig. 7).

3°) *Effet d'injections cumulatives de d-tubocurarine et de succinylcholine ou d'iodure de décaméthonium*

Lorsqu'un poisson vient de subir une injection de *d*-tubocurarine suffisante pour provoquer la disparition presque totale de la décharge

d'origine nerveuse, avec maintien de la réponse directe, on s'attendrait à ce que cette dernière soit réduite par l'injection subséquente de décaméthonium ou de succinylcholine comme il vient d'être dit au 2°. Or il n'en est rien; les expériences présentées dans les fig. 3 et 7 en donnent la démonstration. Tout se passe donc comme si la *d*-tubocurarine exer-

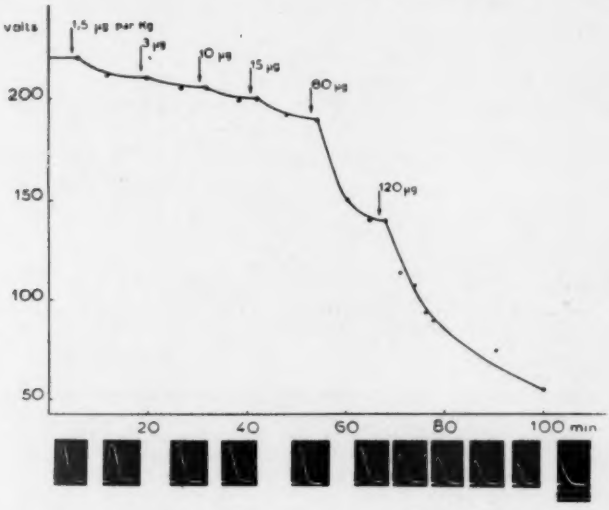

FIG. 6. — *Expérience analogue aux précédentes (3, 4, 5). Ici on essaie des doses croissantes de succinylcholine.*

çait une action protectrice sur les éléments normalement atteints par les ammoniums quaternaires.

4°) *Mesures d'impédance*

Aucune modification de l'impédance, ni de la partie ohmique, ni de la partie réactive, n'a pu être observée au cours des perfusions ou injections soit de *d*-tubocurarine soit des dérivés d'ammoniums quaternaires.

DISCUSSION

De l'ensemble de ces résultats quelques faits nous semblent devoir plus particulièrement être dégagés:

1°) Le fait que l'organe électrique de l'Electrophorus, qui est le seul parmi les organes électriques bien connus, à présenter une réponse directe à la stimulation, soit aussi le seul à présenter en face des curarisants un comportement qui rappelle par toutes ses caractéristiques celui des muscles striés.

2°) Le caractère différentiel de l'action sur l'électroplaxe de la *d*-tubocurarine d'une part et des dérivés d'ammonium quaternaire de l'autre: c'est un fait qui confirme lui aussi l'analogie entre muscle strié et électroplaxes de gymnote. Des expériences ont en effet montré, sur le muscle gracilis du Chat [Paton et coll. (1951), Burns et coll, (1943,

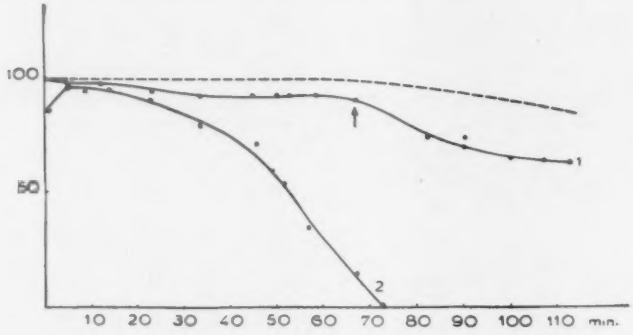

FIG. 7. — *Evolution des amplitudes des réponses directes de trois tranches d'organes perfusées: tracé pointillé, par du serum physiologique - courbe intermédiaire, par une solution de* d- *tubocurarine (46 mg par litre de sérum), puis á partir de la fléche par une solution d'Iodure de décaméthonium (40 mg par litre de sérum) - courbe inférieure, par une solution identique à la précédente d'Iodure de décamethonium. Il est passé au total 450 cm³ de liquide perfusant dans chaque échantillon.*

1951)] que l'action curarisante du décaméthonium s'explique par une dépolarisation que ce poison produit sur la plaque motrice, et aux environs de cette plaque. Ainsi le muscle traité perd son excitabilité directe au moins jusqu'à une certaine distance de la jonction nerf-muscle, de même que l'électroplaxe perd son excitabilité directe.

3°) Il semble enfin que la *d*-tubocurarine (ou le Flaxédil) puisse jouer un rôle protecteur vis-à-vis de l'action des ammoniums quaternaires. Un effet analogue pour le muscle avait été signalé par Paton, Burns et Vianna Dias (1949). Ce fait semblerait prouver que la *d*-tubocurarine se fixe sur une structure attaquée normalement par les substances acétylcholino - mimétiques (effet compétiteur), empêchant ainsi l'action dépolarisante de ces dernières.

Ces expériences confirment pour l'organe électrique la classification des subtances bloquant les transmissions nerf-effecteur, en agents empêchant l'action de l'acétylcholine, et en agents dépolarisant le récepteur. Dans la première catégorie se placent le curare naturel et les curares synthétiques à molécule complexe tels que la *d*-tubocurarine et le Flaxédil, dans la deuxième les dérivés d'ammonium quaternaire [voir Feldberg, (1951) Paton et coll. (1951), Dallemagne (1952, 1953)].

4°) Il a été rappelé dans l'Introduction qu'un des buts importants

de l'étude physiologique de l'électroplaxe est de déterminer dans quelle mesure on peut assimiler celle-ci soit à la fibre musculaire striée, soit à la seule plaque motrice de cette fibre, cette dernière hypothèse ayant été plusieurs fois proposée autrefois. En réalité, chez *Electrophorus* tout au moins, plusieurs données indiquent que l'analogie correcte est avec la fibre musculaire complète: excitabilité directe de l'électroplaxe, transmission de l'excitation de proche en proche à la surface d'une électroplaxe (observation non publiée, 1950), absence de modifications d'impédance après action des dépolarisants présumés de la jonction nerf-électroplaxe. Ce dernier résultat indique en outre comme très probable que l'ensemble des jonctions d'une électroplaxe avec son nerf occupe une surface très faible; on eût sans doute observé sans cela des modifications des propiétés électriques passives au moment de la fixation des substances curarisantes.

RÉSUMÉ

1°) Les échecs anciens pour obtenir l'abolition des décharges par curarisation chez la Torpille, la Raia, le Malaptérure, sont opposés à la possibilité d'obtenir cette curarisation chez le Gymnote (*Electrophorus electricus*).

2°) Ces différences sont mises en parallèle avec l'impossibilité d'obtenir une réponse directe de l'effecteur chez les trois premiers poissons, tandis que deux réponses, directe et d'origine nerveuse, sont dissociables chez le dernier animal.

3°) La *d*-tubocurarine et le Flaxédil ne suppriment que la réponse directe sans modifier l'autre réponse.

4°) L'iodure de décaméthonium et la succinylcholine arrivent par contre à diminuer aussi et à abolir la réponse directe.

5°) L'injection préalable de *d*-tubocurarine et de Flaxédil protège l'effecteur vis-à-vis de l'action des ammoniums quaternaires.

6°) Aucune des substances précédentes ne modifie l'impédance du tissu.

7°) L'analogie entre l'organe électrique de l'*Electrophorus* et la fibre musculaire striée est renforcée par ces constatations.

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THE ACTION OF BRADYKININ, HISTAMINE AND ADENYL COMPOUNDS UPON THE SEMINAL VESICLES OF NORMAL AND CASTRATED RATS *

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IN A RECENT report Pereira (1953) stated that slowly reacting substances like bradykinin (Rocha e Silva, Beraldo & Rosenfeld, 1949; Rocha e Silva, 1951) and substance-U (Beraldo, 1952) might well be taken as a complex mixture of adenosine and histamine. The spasmogenic property of bradykinin, for instance, would depend merely on the presence of histamine in its composition.

As the isolated rat's seminal vesicle is practically insensitive to histamine (Martins, Valle & Porto, 1939) whereas quite sensitive to bradykinin (Picarelli, Branco & Valle, 1951; Prado, Prado, Picarelli & Valle, 1952) it was thought to be a suitable preparation to check Pereira's statement on the smooth muscle stimulating property of bradykinin. Opportunity was taken to verify if the effects of adenylic compounds upon the uterus (Drury, 1936) could be exhibited also by the vesicular musculature.

EXPERIMENTAL

The seminal vesicles were excised from adult normal or castrated rats, separated from the prostate and immersed in warm, oxygenated Ringer-Locke's solution. Either one organ was tested by using a 10 ml bath, or two organs were placed together, in the same 50 ml beaker, for the concomitant registration of their longitudinal contractions. In the first instance a frontal lever was used. When two organs were stu-

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died, each one from a different donor, according to the experimental conditions previously described (Martins & Valle, 1939), then two levers with tangential registration were utilized for the myography. The

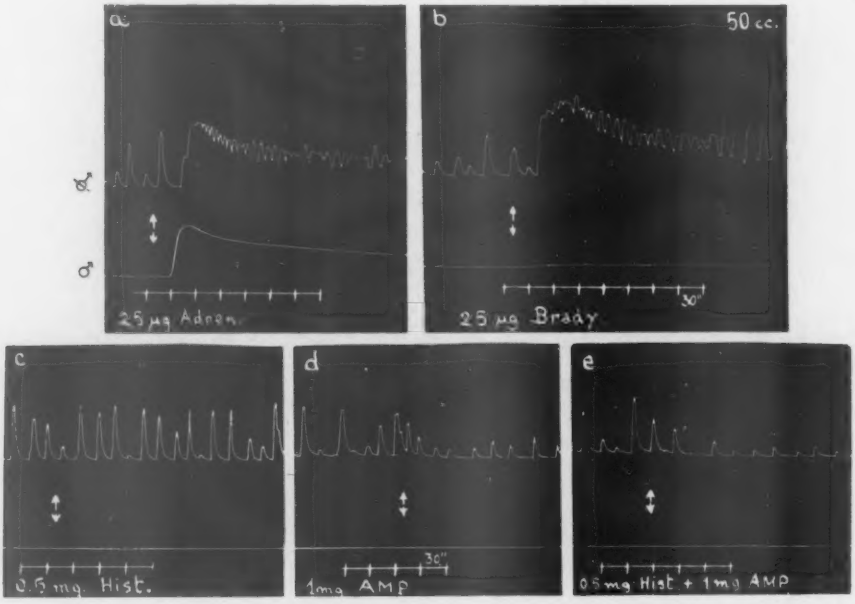

FIG. 1. — *Longitudinal contractions of seminal vesicles from a 48 days castrated (upper tracing) and a normal (lower tracing) rats. Both organs immersed in the same nutritive bath (50 ml of oxygenated Ringer-Locke's solution at 38° C). Time interval 30 sec. a) Epinephrine (25 µg). b) Bradykinin (250 µg). c) Histamine (0.5 mg), practically uneffective. d) Adenosine monophosphate (1 mg), inhibition. e) Histamine (0.5 mg) plus adenosine monophosphate (1 mg) previously mixed before adding to the bath. Inhibition.*

drugs * added to the nutritive bath were histamine (1:50 000-1: 100 000), as diphosphate, adenosine monophosphate (AMP) (1:50 000), adenosine triphosphate (ATP) (1:10 000**) and bradykinin (1:200 000). As control of the reactivity of the organs epinephrine hydrochloride

* Thanks are due to Mrs. Sylvia O. Andrade (Bradykinin, Pool 20X), Eline S. Prado (Hypertensin, EPIIa) and Dr. W. T. Beraldo (Adenosine-monophosphoric acid) for the generous supply of the drugs herein studied.

** Adenosine triphosphate was obtained as the barium salt from Dr. A. Rothschild (Instituto Biológico) and converted into its sodium salt before using. We are also grateful to Ciba S. A. for the supply of adenosine.

(1:1 000 000), arterenol hydrochloride (1:1 000 000) and hypertensin (1:30 000) were employed.

Results and comments

When added to the nutritive bath, bradykinin induces reactions of the seminal vesicles of a different feature according to the hormonal condition of the donors. Organs from normal rats present a single tonic response whereas those from castrated donors exhibit a tonic response

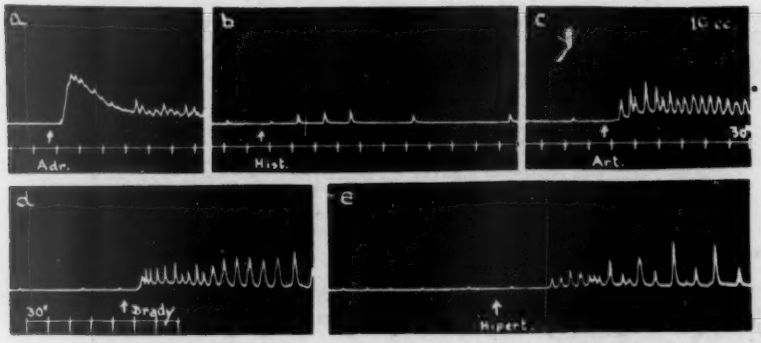

Fig. 2. — *Longitudinal contractions of a seminal vesicle from 45 days castrated rat inmersed in 10 ml of oxygenated Ringer-Locke's solution at 37° C Adr 10 µg of Epinephrine. Hist. 400 µg of Histamine Art. 10 µg Arterenol. Brady 50 µg of Bradykinin (Pool 20X). Hipert 320 µg (0.8 cat unit) of Hypertensin (EPIIa). All reagents but histamine induced strong contraction of the organ. The response was of a tonic-rhythmic type following adrenaline, arterenol and bradykinin. Histamine, even used in dose 8 times higher, only increased the amplitude of the spontaneous contractions. (Time interval 30 sec).*

followed by a series of rhythmic contractions. This particular pattern is similar to that obtained after addition of epinephrine or arterenol (fig. 1, a & b). Influence of castration upon the pharmacological reactivity of rat's male genitals and type of response to several drugs according to the hormonal condition of the donors were already discussed by Martins & Valle (1939), Martins, Valle & Porto (1939).

When hypertensin is used as control agent no response even of a tonic feature is presented by the normal seminal vesicle. The response of the castrate's organ, however, is of the rhythmic type, without residual increase of the tone. This clear cut difference shown in figures 2d, e and 3c, e, deserves mentioning because hypertensin and bradykinin elicit the same type of stimulating action upon the guinea-pig's intestinal musculature (Prado, Prado, Picarelli & Valle, 1952).

Although the stimulating action of histamine upon the castrate's vesicle may be observed in some instances (figs. 2b and 3a), the majority of tests performed with this drug gave negative results. Martins, Valle & Porto (1939) have already stated that histamine induces inconstant effects upon the vasa deferentia, seminal vesicles and prostates

of castrated rats. Upon the same organs from normal or testosterone-castrated-donors they get no definite response with histamine.

The reaction of the castrated rat's seminal vesicles to adenylic acid (AMP) and to adenosine triphosphate (ATP) was of the same type. There is a brief stimulating action characterized by rhythmic decreasing contractions (fig. 3b, f) followed by inhibitory effect of a longer duration. In the present experiments the mixture of histamine with adenylic

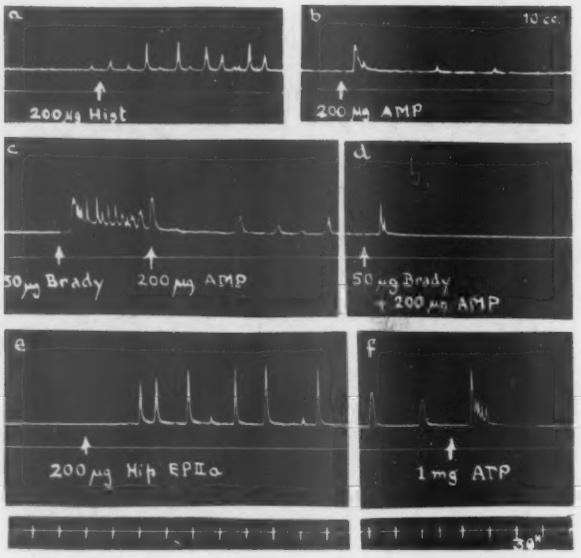

FIG. 3. — *Seminal vesicle from a 51 days castrated rat suspended in a 10 ml Ringer-Locke's bath. The same experimental conditions as in fig. 2. a) 200 µg of Histamine. b) 50 µg of Adenosine monophosphate. c) 50 µg of Bradykinin followed by 200 µg of adenosine monophosphate. d) 50 µg of Bradykinin plus 200 µg of adenosine monophosphate. e) 200 µg of Hypertensin. f) 1 mg of adenosine triphosphate.*

acid, prepared just before addition to the bath, has never elicited a response upon the seminal vesicle of the castrated rat similar to that induced by bradykinin (fig. 1c, d and e).

According to Gillespie (1933) the uterus of the virgin guinea-pig is stimulated by AMP and more by ATP. Cat's uterus is also stimulated by adenylic acid (Floessner, 1934). These authors do not mention if this increase of tone is followed by inhibition. Ewing, Schlenk & Emerson (1949) while analysing the action of isoguanosine upon the guinea-pig's uterus, have observed with adenosine "an increase in tone without much change in the minor rhythmic contractions originally present in some preparations". Preliminary experiments have shown that adenylic compounds do potentiate the stimulating properties of adrenaline, histamine and bradykinin upon the isolated vas deferens of the guinea-pig. This

potentiation may be observed by employing ten times less ATP than adenosine.

From this study one may deduce that the stimulating properties of bradykinin upon the plain muscle are most improbably related to histamine or adenosine.

SUMMARY

Pereira's assumption that the spasmogenic action of bradykinin depends on its histamine content was not corroborated. The isolated rat's seminal vesicle preparation is practically insensitive to histamine whereas presenting a good reactivity to bradykinin. When the organ examined came from a castrated donor, the response to this slowly reacting substance is of a tonic-rhythmic feature like that induced by epinephrine or arterenol.

AMP and ATP may elicit a short stimulating action, the final inhibitory effect being the rule even if bradykinin is still acting.

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metro	m	litro	l	microgramo	μg
centímetro	cm	centímetro cúbico	cm^3	gama	γ
milímetro	mm	mililitro	ml	por ciento	%
micrón	μ	kilogramo	kg	hora	h
milimicrón	mμ	gramo	g	minuto	m
Ångström	Å	miligramo	mg	segundo	s
				milisegundo	ms

Para evitar la confusión derivada de la notación decimal diferente según los países, se adopta el punto decimal y se suprime toda notación entre millares sustituyéndose por un espacio: 10 000 (no 10.000 ni 10,000) —0.90 (no 0,90).

SUMARIO

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